PEPTIDOMIC CHARACTERIZATION AND BIOACTIVITY SCREENING OF LEIURUS ABDULLAHBAYRAMI (SCORPIONES: BUTHIDAE) VENOM

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ABSTRACT

PEPTIDOMIC CHARACTERIZATION AND BIOACTIVITY SCREENING OF LEIURUS ABDULLAHBAYRAMI (SCORPIONES: BUTHIDAE) VENOM

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Animal venoms consist of variety of bioactive molecules, small phenolic compounds to high molecular weight polypeptides, and they exhibit almost limitless biological functions. Scorpion venoms are complex cocktails of toxins from diverse structural origin; they are particularly enriched in bioactive peptides. These peptides gained excellent affinity for their targets through millions of years of evolutionary process. Therefore, scorpion venoms may be defined as naturally selected peptide libraries and this resource offers great promise for development of novel drugs.

Leiurus abdullahbayrami, a recently described scorpion species of Turkey, produces one of the most potent venoms among venomous animals that may cause severe symptoms in envenomed victims. The remarkable strength of its venom suggests the presence of highly potent peptide toxins.

The aim of this work was to characterize *L. abdullahbayrami* venom, mainly

focusing on the peptide content and to screen its bioactivity. For this reason biochemical and peptidomic approaches were followed as well as cellular assays to screen bioactivity. The protein content of the venom was found 54%. The comparative electrophoretic profiling with three distinct techniques highlights the high abundance of peptide neurotoxins and indeed low amounts of high molecular weight proteins in the venom. Microfluidic capillary electrophoresis not only provided superior resolution but also had the advantage of less sample requirement over other methods. 45 unique peptide masses between 1-7 kDa were detected, supporting the presence of both short-chain and long-chain neurotoxins as well as non-disulfide bridged peptides as expected. A significant proliferative effect of crude venom was seen on MCF-7 cells. The antimicrobial potential of the venom was screened on bacterial and fungal strains, and a promising activity was detected on gram negative bacteria.

The venomic components responsible for the observed activities of the present study may be determined, individually, by further investigations. The bioactive molecules might be used as scaffolds for the development of novel drugs, through advanced characterization and molecular engineering endeavors.

The results of this study were published in article: Erdeş et al.: Characterization of *Leiurus abdullahbayrami* (Scorpiones: Buthidae) venom: peptide profile, cytotoxicity and antimicrobial activity. *Journal of Venomous Animals and Toxins including Tropical Diseases* 2014 20:48. (doi:10.1186/1678-9199-20-48)

Keywords: Venomics, peptidomics, peptide, toxin, scorpion venom, *Leiurus abdullahbayrami*, cytotoxicity, cell proliferation, antimicrobial, cancer, ion channels

ÖZ

LEIURUS ABDULLAHBAYRAMI (SCORPIONES: BUTHIDAE) VENOMUNUN PEPTİDOMİK KARAKTERİZASYONU VE BİYOAKTİVİTE TARAMASI

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Hayvan venomları küçük fenolik bileşiklerden yüksek moleküler ağırlığa sahip polipeptitlere kadar değişkenlik gösteren ve neredeyse sınırsız biyolojik fonksiyona sahip biyoaktif moleküllerden oluşmaktadır. Akrep venomları özellikle biyoaktif peptitler açısından zengin olmakla birlikte, farklı yapısal kökenden gelen birçok toksinin kompleks karışımı niteliğindedirler. Bu peptitler milyonlarca yıllık evrimsel süreçte hedeflerine karşı mükemmel afinite kazanmışlardır. Bu nedenle, akrep venomları doğal seçilim yoluyla oluşmuş peptit kütüphaneleri olarak tanımlanabilir ve bu kaynak yeni ilaçların geliştirilmesi açısından büyük umutlar vaat etmektedir.

Türkiye'de yeni tanımlanmış bir akrep türü olan *Leiurus abdullahbayrami*, zehirli hayvanlar arasında en etkili venomlardan birine sahip olup, sokulma vakalarında kurbanlarda ciddi semptomlara sebebiyet vermektedir. Venomunun bu kayda değer gücü, içerisinde barındırdığı yüksek etkili peptit toksinlere dikkat çekmektedir.

Bu çalışmanın amacı *L. abdullahbayrami* venomunun peptit içeriğine odaklanılarak karakterize edilmesi ve biyoaktivitesinin taranmasıdır. Bu amaçla biyokimyasal ve peptidomik yaklaşımlar takip edilmiş aynı zamanda da biyoaktivite taranması için hücresel sistemler kullanılmıştır. Venomun protein miktarı %54 olarak bulunmuştur. Üç farklı teknik ile yürütülen karşılaştırmalı elektroforetik profilleme ile venomda yüksek miktarda peptit nörotoksinin ve çok az miktarda yüksek moleküler ağırlığa sahip proteinin varlıkları aydınlatılmıştır. Mikroakışkan kapiler elektroforez diğer elektroforetik metotlara göre daha az örnek gereksinimi sunmakla kalmayıp, daha üstün ayırma gücü de sağlamaktadır. 1-7 kDa arasında 45 adet özgün peptit kütlesinin tespiti kısa-zincir, uzun-zincir ve hatta disülfit bağı içermeyen peptitlerin beklendiği üzere venomdaki varlığını desteklemiştir. Venomun MCF-7 hücreleri üzerinde anlamlı proliferatif etkisi görülmüştür. Venomun antimikrobiyal potansiyeli bakteriyel ve fungal suşlar üzerinde taranmış ve gram negatif bakteriler üzerinde ümit veren sonuçlar gözlemlenmiştir.

Bu çalışmada ortaya koyulan aktivitelerin hangi venomik bilşenden kaynaklandığı ilerdeki araştırmalar ile belirlenebilir. İleri karakterizasyon ve moleküler mühendislik çalışmaları sayesinde belirlenen biyoaktif moleküller, yeni ilaçların geliştirilmesinde altyapı teşkil edebilir.

Bu çalışmanın sonuçları belirtilen makalede yayınlanmıştır: Erdeş et al.: Characterization of *Leiurus abdullahbayrami* (Scorpiones: Buthidae) venom: peptide profile, cytotoxicity and antimicrobial activity. *Journal of Venomous Animals and Toxins including Tropical Diseases* 2014 20:48. (doi:10.1186/1678-9199-20-48)

Anahtar Kelimeler: Venomik, peptidomik, peptit, toksin, akrep venomu, *Leiurus abdullahbayrami*, sitotoksisite, hücre proliferasyonu, antimikrobiyal, kanser, iyon kanalları

In Memory of my Beloved Grandparents,

Güler Mükerrem Demircioğlu

and

Mustafa Nihat Demircioğlu

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ACE	Angiotensin-converting enzyme
GLP-1	Glucagon-like peptide-1
nAchRs	Nicotinic acetylcholine receptors
UV	Ultraviolet
DNA	Deoxyribonucleic acid
Na^+	Sodium ion
K^+	Potassium ion
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
NDBPs	Non-disulfide bridged peptides
Da	Dalton
kDa	Kilodalton
ICK	Inhibitory cysteine knot
AMP	Antimicrobial peptide
MW	Molecular weight
MIC	Minimum inhibitory concentration
cDNA	Complementary deoxyribonucleic acid
IL-10	Interleukin-10

TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
MMP-2	Matrix metalloproteinase-2
mm's	Millimeters
HPLC	High performance liquid chromatography
SEC	Size exclusion chromatography
RP	Reversed phase
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
MCE	Microfluidic capillary electrophoresis
LC/MS	Liquid chromatography / mass spectroscopy
TOF	Time of flight
ESI	Electrospray ionization
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
Pen-Strep	Penicillin - Streptomycin
DMSO	Dimethyl sulfoxide
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-

	Tetrazolium-5-Carboxanilide
EDTA	Ethylenediaminetetraacetic acid
PBS	Phosphate buffered saline
ER	Estrogen receptor
PCR	Polymerase chain reaction
DC	Direct current
dH_2O	Deionized water
ANOVA	Analysis of variance
Log	Logarithm
ACN	Acetonitrile
TFA	Trifluoroacetic acid
NC	Negative control
Eto	Etoposide
CV	Crude venom
DTT	Dithiothreitol

CHAPTER 1

INTRODUCTION

1.1 Toxin, Poison and Venom

Toxins are biomolecules produced in living organisms that have diverse biological effects on the exposed organism's metabolic pathways or briefly poisonous substances originated from biological systems. Toxins are evolved in numerous organisms belonging all domains of life. From primitive bacteria to higher eukaryotic organisms, a wide range of species produces toxins. Toxins could be used as either a defense mechanism or a preying strategy (Meier & White 1995).

There are many misuse and misunderstanding on the concepts of toxin, poison and venom. In daily language toxic materials such as synthetic compounds, pollutants or heavy metals are considered as toxins where they are in actuality toxicants. As it was aforementioned before, all toxins are originated from biological sources. Toxins could be classified in groups depending on different parameters and characteristics. An accepted classification handles toxins in terms of structure as non-peptide toxins and peptide toxins. Microbial species, plants and fungi are the main reserve of non-peptide toxins. They are usually orally active and act as poisons in case of swallowing. The largest portion of the complex mixture of animal venoms' consisting of peptide toxins. This complex mix are injected to the prey or threat through a bite, sting or by any specialized apparatus via subcutaneous, intradermal or intravenous routes and so called venoms. Organisms that synthesize toxins and consists them inside theirs bodily

compartments or secrete them to near adjacencies called as poisonous. On the other hand, organisms that have evolved with a specialized venom gland and an apparatus to inject their toxins are classified as venomous. All venomous organisms are classified under Animalia kingdom, so that generally called as venomous animals. However poisonous animals also exist. Toxins originated from poisonous and venomous species are significant natural resources to screen indefinite bioactive properties, which they exhibit (Meier & White 1995).

1.2 Animal Venoms

Animal venoms were evolved through the evolutionary process to capture prey and for defensive measures against threats. Variety of classes under Animalia kingdom such as Mammalia (mammalians), Reptilia (reptiles), Actinopterygii (ray-finned fishes), Chondrichthyes (cartilaginous fishes), Cephalopoda (cephalopods), Anthozoa (anthozoans), Cubozoa (box jelly fishes), Scyphozoa (true jelly fishes), Gastropoda (gastropods), Insecta (insects), Chilopoda (centipedes) and Arachnida (arachnids) comprises venomous animals. Animal venoms are complex mixtures of both inorganic and organic molecules. These are namely ions, small organic molecules such as biogenic amines and polyamines, peptides, enzymes and proteins. The content and ratio of venom is variable between different classes, genus and even species. Sex, pregnancy in female specimens, age and habitat also affect the composition of the venom. However, the major portion of animal venoms consist of peptides and proteins, which show diverse bioactive properties (Lewis & Garcia 2003).

1.2.1 Mammalian Venoms

The prominent instances of venomous mammals are male platypuses

(*Ornithorhynchus anatinus*). These egg-laying mammals synthesize defensin-like proteins in their crural venom glands and inject their venom by their spur as a defense mechanism (de Plater et al. 1995; Whittington et al. 2008). Shrews evolved with modified saliva glands and grooved teeth to produce and inject their venoms (Martin 1981). Arguably, slow and pygmy lorises (*Nycticebus* genus) are considered to be venomous primates. The venom synthesized in brachial glands and become activated upon contact with their saliva after self-grooming (Hagey et al. 1996).

1.2.2 Reptile Venoms

Venomous reptiles have evolved saliva glands into intracranial venom glands and many of them contain grooved fangs or teeth to deliver their toxic cocktail to prey/victim. Probably the class Reptilia embraces the most familiar venomous animals, snakes. Although most of the snake species are non-venomous or slightly venomous; Elapidae (elapids), Viperidae (viperids) and Colubridae (colubrids) families contain venomous species. Elapids involve cobras and sea snakes and venom mainly consist of neurotoxins. Viperids are the family that embraces vipers and rattle snakes, which are producing highly potent proteolytic enzymes in theirs' venom glands. Colubrids were initially thought to be non-venomous, yet relatively recent researches changes this consensus (Tu 1973; Fry et al. 2003; Koh et al. 2006).

Snake venoms are the most studied group and the one having highest number of licensed drugs among animal venoms. The first success story of development of a drug from a previously discovered toxin is captopril. This drug was firstly isolated as a Bradykinin-potentiating peptide from *Bothrops jararaca* venom, which is actually an Angiotensin-converting enzyme (ACE) inhibitor. The modified and synthetic version of this venomic component is currently in use as a hypotensive drug. Moreover, many other ACE inhibitors are under development based on the

active Phe-Ala-Pro core of the captopril. Other than hypotensive drugs, two antiplatelet agents were introduced from snake venoms. These are Tirofiban and Eptifibatide, released in the commercial names of Aggrastat® and Integrillin®, respectively. Tirofiban and Eptifibatide are disintegrins that inhibit the binding of fibrinogen to its receptor, preventing the platelet aggregation. The R-G-D and K-G-D tripeptide sequence motifs of these peptides are responsible for the inhibition at molecular level (Koh & Kini 2012).

Other than snakes, reptiles such as Gila monster (*Heloderma suspectum*) and Komodo dragon (*Varanus komodoensis*) have venom glands and use their venom for preying and defensing strategy. A glucagon-like peptide-1 (GLP-1) agonist, Exendin-4, was discovered from the venom of *H. suspectum* (Eng et al. 1992; Goke et al. 1993). The synthetic version of this peptide named Exenatide released to market in commercial name of Byetta. Byetta currently used in the patients diagnosed with diabetes mellitus type-2, being one of the well-known peptide drugs (Bond 2006).

1.2.3 Fish Venoms

Ray-finned fishes, a subclass of bonny fishes, accommodate venomous families such as Trachinidae (weever fishes), Synanceiidae (stone fishes), Scorpaenidae (scorpion fish and lion fish), Uranoscopidae (stargazers) and Batrachoididae (toadfish). These have specialized venom glands and spiky fins to produce and inject their venom. Moreover, cartilaginous fishes such as certain species of stingrays (Myliobatoidei), dogfish sharks (Squalidae) and chimaeras (Chimaeriformes) produce venoms. Stingrays use long and barbed stingers to inject their venom, whereas dogfish sharks and chimaeras have venomous dorsal spines (Smith & Wheeler 2006). Envenomation cases caused by venomous fishes are generally painful for the victim, but rarely lethal.

1.2.4 Cephalopod Venoms

The only venomous cephalopod among many of poisonous one is blue-ringed octopus (*Hapalochlaena* genus). These octopuses do not own a specialized venom gland, but host a symbiotic bacteria in theirs' salivary glands to produce toxins (Hwang et al. 1989). This bacterial species synthesize the toxin, Tetrodotoxin, which blocks voltage gated Na^+ channels in nanomolar concentrations (Sheumack et al. 1978). Therefore blue ringed octopus envenomation may cause severe symptoms such as paralysis and respiratory arrest.

1.2.5 Anthozoan Venoms

Sea anemones belonging the class of Anthozoa are one of the main suppliers of peptide toxins. These marine creatures evolved with an exciting venom-injection apparatus called nematocysts. These harpoon-like organs are triggered by physical stimuli and attached to prey/threat to inject toxic cocktail of toxins. Previously, for instance, three polypeptide neurotoxins: AETX I, AETX II and AETX III were investigated from sea anemone species *Anemonia erythraea* venom (Shiomi et al. 1997). At the same year, a polypeptide named halcurin was isolated from the venom of *Halcurias* genus (Ishida et al. 1997). Both of these peptides were observed with selective toxicity to crabs, where no hemolytic activity was seen on mammalian erythrocytes.

1.2.6 Jelly Fish Venoms

Jellyfishes are responsible for many envenomation cases at coastal areas, especially in the course of summer seasons according to the increased human interaction. Most of the jellyfishes cause mild symptoms upon stinging, unlike some exceptions like the Box jellyfishes. For instance the species *Chironex*

fleckeri of Indo-Pacific marine ecosystem has the most painful sting among jellyfishes, which can cause severe symptoms. Contrary to true jellyfishes, they have functional eyes that may associate with the use of theirs' venom in active hunting strategy. Two protein toxins with hemolytic activity were isolated from the venom of *Carybdea alata* and were named as CaTX-A and CaTX-B (Nagai et al. 2000). Recent venom characterization studies on *C. fleckeri* lead to identification of novel toxins in protein structure, which act on cardiovascular system: CfTX-1 and CfTX-2. Other than these, toxins CfTX-A and CfTX-B with hemolytic activity were also discovered from the same species (Brinkman et al. 2014).

1.2.7 Cone Snail Venoms

Cone snails are marine gastropods considered under the genus *Conus*. These species evolved with venom glands and a specialized harpoon-like radula for venom delivery. Cone snails are carnivores and normally use their venom for paralyzing their preys (Kohn 1956). However upon threatening they may use it for defense, which can cause severe envenomation cases in humans. The prominent component of cone snail venom is peptide toxins, where low molecular weight components and proteins also present (Gray & Olivera 1988). They are named as conotoxins and classified in groups according to their mode of action. α-conotoxins (alpha-conotoxins) inhibit the nicotinic acetylcholine receptors (nAchRs), δ-conotoxins (delta-conotoxins) and μ-conotoxins (mu-conotoxins) act on voltage-gated Na^+ channels, κ-conotoxins (kappa-conotoxins) block the voltage-gated K^+ channels and ω-conotoxins (omega-conotoxins) inhibit the N-type neuronal voltage-sensitive Ca^{2+} channels (Olivera et al. 1991; Shon et al. 1998; Leipold et al. 2005). In the past decade a synthetic analog of a ω-conotoxin, MVIIA (SNX-111), from *Conus magus* was developed as a drug against chronic pain in commercial name Prialt (Ziconotide) (Bowersox & Luther 1998; Wang et

al. 2000). Therefore, cone snail venoms may be considered as a great resource of bioactive peptides, which may be further screened to improve novel peptide drugs.

1.2.8 Insect Venoms

Insects are the most diverse class in the terrestrial ecosystems having more than a million described species. The order of Hymenoptera under Insecta class involves venomous insects such as bees, wasps and ants. Bee venom is a cocktail of variety of peptide and proteins. Beside the high content of well-known peptide toxins such as melittin and apamin, phospholipase A2, hyaluronidase-family enzymes and small organic molecules such as histamine compose the venom content (Habermann 1972; Banks et al. 1979). The Formicinae subfamily of ants has mostly formic acid in their venom. Fire ant (*Solenopsis* genus) venoms were initially though to be composed of alkaloids, merely. However later research on the venom of a fire ant, *Solenopsis invicta*, show the protein components presence similar to other Hymenoptera species (Baer et al. 1979).

1.2.9 Centipede Venoms

Subphylum of Myriapoda embraces the classes Diplopoda (millipedes) and Chilopoda (centipedes). Unlike their decomposer relatives, millipedes; centipedes are strict carnivores. They use their venom for preying strategy and it is secreted from strong venom claws. Although many of the arthropod venoms are well studied, centipede venoms are still keeps its mystery. Up to date researches showed the presence of neurotoxic, cytotoxic, myotoxic and hemotoxic components in centipede venoms. Most of the venomic content is composed of enzymes, but low molecular weight fractions as well as peptides were also identified (Undheim & King 2011).

1.3 Arachnids, Scorpions and Scorpion Venoms

1.3.1 General Characteristics of Arachnids

Arachnida class belongs to phylum of Arthropoda and subphylum of Chelicerata, phylogenetically. Currently living arachnids were evolved about four hundred millions years ago and originated from sea ecosystems. Arachnids could be easily differentiated from insects by their eight-legged anatomy and distinguishable mouthparts called chelicerae (Reptile Expert). Arachnids include harvestmen, mites, sun spiders (a.k.a. camel spiders, Order: Solifugae), pseudoscorpions, spiders and scorpions etc. Pseudoscorpions, scorpions and spiders exhibit specialized venom glands. The venom gland and duct located in the chelipeds (pincers) of pseudoscorpions. Spiders use modified chelicerae, so called fangs, to transfer venom produced in glands located inside cephalothorax. Scorpions have venom glands in telson, at distal metasoma, and use adjacent sting for delivery. Morphology of the venom duct on sting and the chelicerae of a scorpion can be seen at Figure 1.1.

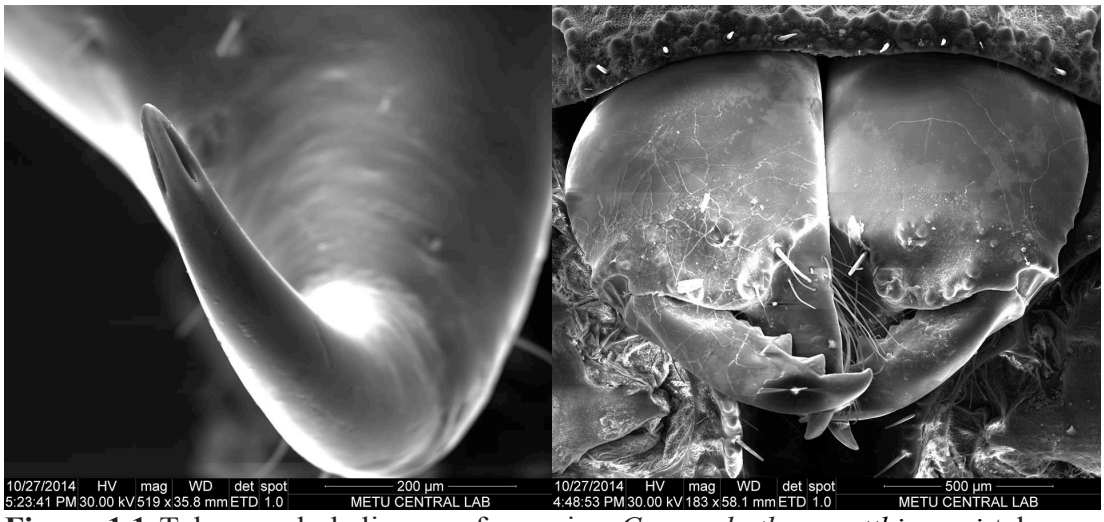

Figure 1.1 Telson and chelicerae of scorpion *Compsobuthus matthiesseni* taken by Scanning Electron Microscopy (SEM). The venom duct on telson magnified 519 x (left) and chelicerae magnified 183 x (right).

1.3.2 The Biology and Distribution of Scorpions

Scorpions consist of fourteen families and more than 1700 described species over the world, adapted diverged habitats over continents except Antarctica (Ortiz et al. 2015). This order of animals can be encountered in desert, steppe, forest, chaparral, mountain and even isolated cave ecosystems. All scorpions are nocturnal and carnivores. The body of scorpions is dissected in two parts: prosoma and opisthosoma where opisthosoma further divided into mesosoma and metasoma. These parts include cephalothorax, abdomen and tail, respectively. Scorpions have eight legs and a pair of pincers called chelipeds. As an arthropod, scorpions have open circulatory system and the respiration is achieved through book lungs instead of trachea. Scorpions are not egg-layers. The fertilized eggs are carried by female scorpions to give them birth. The female scorpions are known to carry newborn scorpionlings at their back. This may continue until juveniles undergo a couple of molting. This care of mother to young is uncommon in arthropods. Being cold-blooded animal, scorpions are usually inactive during cold season. Interestingly, an organic compound found in the cuticle of scorpions causes greenish illumination of the exoskeleton upon excitation at near UV spectrum. This compound is a coumarin derivative, hymecromone (or 4-methyl-7-hydroxy-coumarin), which is before known merely from plant sources (Figure 1.2 inset). This property is present in all scorpion species, yet first instar juveniles and freshly molted scorpions do not exhibit fluorescence. It has been hypostasized as evolvement for sun blocking during their transition from sea to land. This may provided them an advantage to prevent DNA damage due to UV exposure. To our benefit, illumination of scorpions under UV facilitates collection of specimens during field studies at nighttime (Frost et al. 2001; Özkan & Karaer 2007).

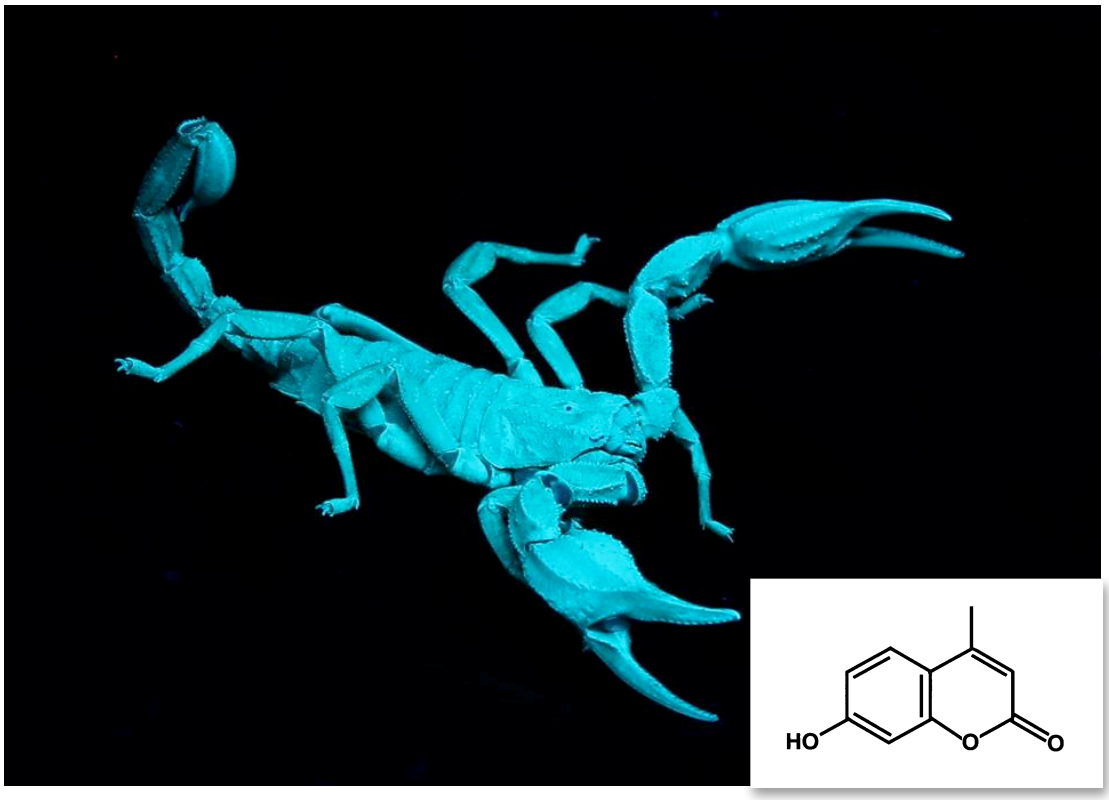

Figure 1.2 *P. kraepelini* illuminated with UV. 4-methyl-7-hydroxy-coumarin molecule (inset). (Photo courtesy of Naşit İğci)

1.3.3 Arachnid Venoms

Arachnid venoms are considered to be one of the richest bioactive peptide libraries among arthropods, since their complex venom cocktail involves many peptide toxins (Possani & Rodríguez de la Vega 2006). Venoms of spiders and scorpions mainly include inorganic salts, amino acids, nucleotides, small organic molecules such as biogenic amines, polyamines, peptides and proteins (Quintero-Hernández et al. 2013; Rash & Hodgson 2002). Neurotoxins in peptidic structure comprise the major portion of the venom and more than a hundred different peptides may be found in scorpion venom (Delepierre et al. 1999). This number may come up to a thousand in the spider venoms. Peptide neurotoxins of arachnid

venoms are classified into four groups with respect to their target ion channels. These groups are namely Na^+, K^+, Ca^{2+} and Cl^- ion channel inhibitors (Srinivasan et al. 2001). In the course of four hundred million years of evolutionary process, the neurotoxins of arachnid venoms gained excellent affinity and specificity against their target ion channels that make them highly potent ion channel blockers. (Tan et al. 2006) Beside the ion channel inhibitor neurotoxins, arachnid venoms also contain short polypeptides that exhibit antimicrobial effects against various microorganisms. Since arachnids' diet involves mainly insects, presence of peptides with insecticidal activity is not indeed surprising (De Lima et al. 2007). Therefore, arachnid venoms are a great natural resource for bioactive peptide screening. Due to their remarkable functionality and structure, venomic peptide toxins offer invaluable platforms to develop novel therapeutic and diagnostic approaches (Escoubas & Rash 2004).

1.3.4 Scorpion Venoms

As in the case of arachnid venoms, the majority of the scorpion venom consists of peptides. These peptides may be considered in two groups: peptides with disulfide bridges and peptides without disulfide bridges (a.k.a. non-disulfide bridged peptides, NDBPs). Peptides with disulfide bridges are cysteine rich cationic peptides that are either Na^+, K^+, Ca^{2+} or Cl^- ion channel inhibitors, which are acting as neurotoxins. These further can be divided into two groups structurally: short-chain neurotoxins and long-chain neurotoxins. Short-chain neurotoxins are about 3 kDa to 5 kDa, where long-chain neurotoxins have a molecular weight of 6-8 kDa. Short-chain neurotoxins comprise the potassium ion channel flux modulators, while long-chain neurotoxins involve modulators of sodium ion flux through cell membranes. This bimodal distribution of peptide masses is a characteristic of an arachnid venom, so that also scorpion venoms. Short-chain neurotoxins share a common structural motif called as inhibitory cysteine knot

(ICK). This motif is conserved through the evolutionary process from cone snails to arachnids (Figure 1.3). ICK motif highly strengths the peptidic structure that these peptides are significantly resistant to low pH, high temperatures and even protease attacks (King & Hardy 2013; Daly & Craik 2011). Thus, the structural stability of ICK motif containing peptides is unique among natural polypeptidic molecules. On the other hand, non-disulfide bridged peptides in scorpion venoms were identified in relatively recent time. These peptides generally present a molecular weight between 1-5 kDa, yet mostly accumulated to 1-3 kDa. NDBPs were shown to exhibit antimicrobial, immunomodulatory, Bradykinin-potentiating and hemolytic activities (Zeng et al. 2005).

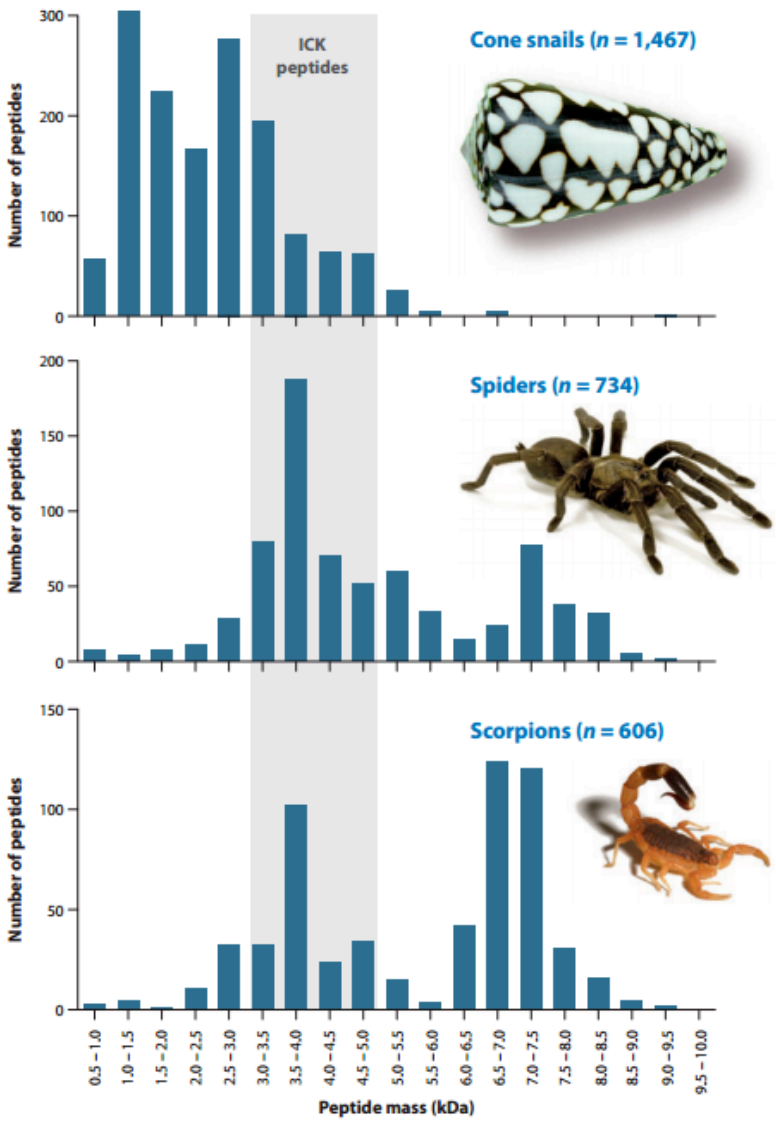

Figure 1.3 Molecular weight distribution histogram of previously discovered venomic peptides of cone snails, spiders and scorpions. The bimodal distribution trend of short-chain and long-chain neurotoxins of arachnid venoms can be seen. (King & Hardy 2013)

1.3.5 Previously Identified Peptide Toxins from Scorpion Venoms as Drug Candidates

More than nine hundred scorpion venom peptides have been identified, so far,

according to UniProt database (UniProt). The peptides were shown to present antibacterial, antifungal, antiviral, antimalarial, immunomodulatory, Bradykinin-potentiating, anticancer and autoimmunity-suppressing activities. Antimicrobial peptides (AMPs) can be found in diverse variety, from bacteria to higher eukaryotic organisms. The roles of AMPs as a venom component are still unclear, yet a large number of them were isolated from scorpion venoms. Some AMPs discovered so far are hadrurin from *Hadrurus gertschi*, pandinins from *Pandinus imperator* and parabutoporin from *Parabuthus schlechteri*. Most of the AMPs from scorpion venoms are classified into NDBPs group, structurally. These peptides generally show low minimum inhibitory concentration (MIC) values against even multi-resistant pathogenic strains. However, the major disadvantage of venomic antibacterial peptides is the high cytotoxic and hemolytic activity on mammalian cells. These peptides are trying to be engineered to overcome this phenomenon. The NDBPs that show antibacterial activity are generally also effective on pathogenic fungal species such as *Candida* species, *Cryptococcus neoformans* and *Aspergillus fumigatus*. For instance; Pandinin2 and parabutoporin have been shown to inhibit growth of *Candida albicans* and *Saccharomyces cerevisiae*, respectively, at micromolar concentrations. Beside the antibacterial and antifungal peptides, antimalarial peptides against *Plasmodium falciparum* were discovered from scorpion venoms. Two peptides that follow the non-disulfide bridged structure motif were isolated by screening of cDNA library obtained from venom gland of *Mesobuthus eupeus*. The peptides named meucin-24 and meucin-25 are capable of eliminating *P. falciparum* infection completely at a dose of 10 μM in 72 hours without any toxicity to host cells. On the other hand, a limited number of venomic peptides with antiviral activity have been encountered. An example to antiviral scorpion venom peptide could be the Hp1090 from *Heterometrus petersii*. Again at micromolar concentrations, Hp1090 was shown to be able to inhibit amplification of Hepatitis C virus with disturbing its phospholipid membrane. Many studies on peptides derived from scorpion venoms are highlighted the immunomodulatory functions of them. Some of these

scorpion venom peptides are acting as immunostimulatory agents to increase secretion of pro-inflammatory cytokines and some were shown to induce secretion of immunosuppressant cytokines such as IL-10. A peptide that was isolated from *Tityus serrulatus*, TsV, activates complement system as well as interacts with Toll-like receptors TLR2 and TLR4 results in the activation of NF-κB pathway. Bradykinin is a vasodilator peptide expressed endogenously, having an essential role in the decreasing blood pressure. Angiotensin-converting enzyme (ACE) cleaves and inactivates Bradykinin activity, so that a significant target for treatment of the hypertension. K_{12} peptide isolated from the venom of *Buthus occitanus* shows Bradykinin-potentiating activity by directly inhibiting ACE. Other peptides from scorpion toxins were identified to potentiate Bradykinin activity, however they do not act directly on ACE and probably act on endothelial function. The Kv1.3 ion channels take the primary role in T-cell proliferation and activation. Since scorpion venoms are rich in Kv ion channel inhibitor peptides, they are invaluable sources for the drug development for autoimmune disorders and lymphoproliferative diseases. Margatoxin (Figure 1.4, right) from *Centruroides margaritatus* and charybdotoxin of *Leiurus quinquestriatus* are well known instances of Kv1.3 inhibitor venomic peptides. Maybe the most interesting scorpion venom peptide with anticancer effect is chlorotoxin from *L. quinquestriatus*. Chlorotoxin (Figure 1.4, left) was initially thought to be inhibitor of small-conductance Cl^- channels, merely. However further research identified its unique capacity to bind matrix metalloproteinase-2 (MMP-2). By this, chlorotoxin is able to inhibit metastasis of glioma cells, *in vitro*. More interestingly, chlorotoxin-MMP-2 interaction is specific for glioma cells but not healthy neurons or glial cells. Thanks to this specific interaction, fluorescent dye conjugated chlorotoxin is now developing as a diagnostic tool behalf of surgeons to confirm dispersion of the tumor burden. Mentioned examples on use of venomic peptides as a part of therapeutic and diagnostic approaches highlights the significance and potential of scorpion venoms. This natural resources may offer scaffolds for the development of numerous novel peptide drugs on the coming

decades (Ortiz et al. 2015; Veiseh et al. 2007; Chi et al. 2010; DeBin et al. 1993; Gimenez-gallego et al. 1988).

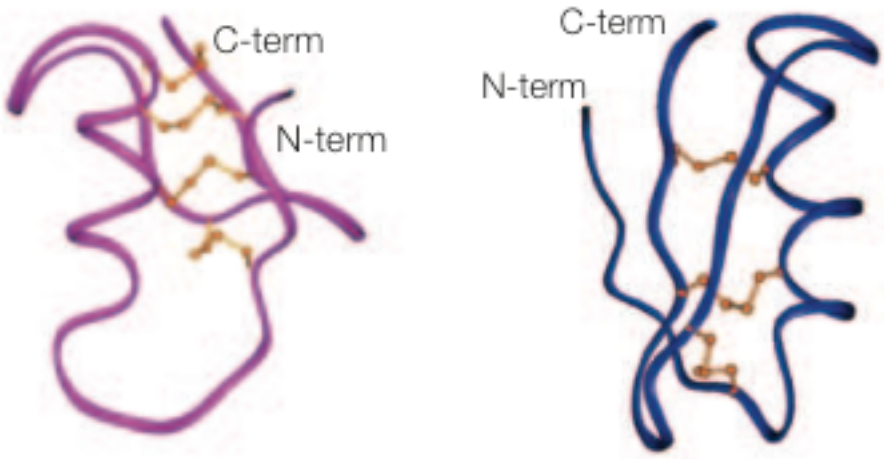

Figure 1.4 The structures of chlorotoxin (left) and margatoxin (right). Cysteine bridges are shown in yellow (Lewis & Garcia 2003).

1.4 Scorpions of Turkey

According to the latest records, Turkey has twenty-eight scorpion species under four families and thirteen genera (Caliskan 2014; Tropea et al. 2014). These four families are Buthidae, Scorpionidae, Iuridae and Euscorpiidae. The Buthidae family are presented by seven genera: *Androctonus*, *Buthacus*, *Compsobuthus*, *Hottentotta*, *Leiurus*, *Mesobuthus* and *Orthochirus*. *Scorpio* genus is the only member of Scorpionidae family in Turkey. Genera *Calchas*, *Neocalchas*, *Iurus* and *Protoiurus* belong to the Iuridae family. The Euscorpiidae family comprises also a single genus in Turkey, *Euscorpius*.

1.4.1 Buthidae Family

Most of the scorpion species are mildly venomous and may be considered harmless to mammals and so human beings, except the allergic responses of some individuals. However, Buthidae family includes scorpions, which are the most medically important and may cause severe envenomation cases upon stinging. In consensus; the genera *Androctonus*, *Hottentotta*, *Leiurus*, *Parabuthus* from old world and *Centruroides*, *Tityus* from new world buthids thought to be ones that produce most potent venoms. (Ozkan et al. 2011) Buthid stings are still very rarely lethal to healthy adults, but may be deadly to infants, children, elders and the ones that suffer chronic heart diseases (Sahin et al. 2014). The scorpions belonging to the Buthidae family evolved with mammal-specific neurotoxins, which may responsible for the severe clinical profile of envenomed victims (Chippaux & Goyffon 2008). Buthidae is the largest family of scorpions embraces more than eight hundred species. The buthid species of Turkey are *Androctonus crassicauda* (Figure 1.5a), *Buthacus macrocentrus* (Figure 1.5c), *Compsobuthus matthiesseni*, *Compsobuthus schmiedeknecthi*, *Hottentotta saulcyi*, *Leiurus abdullahbayrami*, *Mesobuthus gibbosus* (Figure 1.5b), *Mesobuthus eupeus*, *Mesobuthus nigrocinctus*, *Mesobuthus caucasicus*, *Mesobuthus phillipsii* and *Orthochirus zagrosensis*. The only buthid species greatly distributed through the more humid Western/Southern part of Anatolia as well as semi-arid steppe areas of Central Anatolia is *M. gibbosus* and responsible for most of the envenomation cases at seaside locations during summertime. *M. eupeus* also recorded from Central Anatolia, but do not spread through the Western and Southern parts. Rest of the Buthidae family members live in semi-arid and arid steppes of the Eastern and Southeastern Anatolia, as a characteristic of buthids. *A. crassicauda*, *L. abdullahbayrami*, *B. macrocentrus* and *Mesobuthus* species are considered to be medically important scorpion species of Turkey (Ozkan et al. 2007). However there are limited numbers of research on *H. saulcyi* envenomation records, probably due to its narrow dispersion, and which is possibly health threatening as

well. *A. crassicauda* is the scorpion species that causes many of the severe envenomation cases and the species mostly responsible for death incidences at Turkey due to scorpion stings. The meaning of the Latin scientific name *Androctonus crassicauda* is Man-killer fat-tailed (Andro-ctonus crassi-cauda) that originated from its notoriety. This species considered having less potent venom than that of *Leiurus* genus, yet *A. crassicauda* tend to live close to human settlement (Caliskan 2014).

1.4.2 Scorpionidae Family

Scorpio maurus (Figure 1.5d) is the single genus and species of Turkey belonging Scorpionidae family. *S. maurus* is the first scientifically described scorpion species in the world. This species have many subspecies and distributed through North Africa and Middle East and it is recorded in Southeastern Anatolia. *S. maurus* is a burrow-dwelling species and digs characteristic nests beneath soil (Çolak & Karataş 2013). Its venom considered being innocuous to humans.

1.4.3 Iuridae Family

Iuridae family dispersed through a limited geography from Caucasus to Greek Islands, passing through the Anatolia and represented by nine species under four genera in Turkey. Many members of this family, except some of the *Calchas* species, appear in relatively humid forest habitats unlike buthids and *S. maurus*. Iurids of Turkey are *Calchas anlasi*, *Calchas birulai*, *Calchas kosswigi*, *Calchas nordmanni*, *Neocalchas gruberi*, *Iurus kinzelbachi*, *Protoiurus asiaticus*, *Protoiurus kraepelini* (Figure 1.5d) and *Protoiurus kadleci* (Soleglad et al. 2012; Yağmur et al. 2013). They very rarely attempt to sting, even when aggressed by a threat. Their venom is not potent indeed and not much worse than a bee sting for a

healthy human. They generally use their strong chelipeds to catch prey. *Iurus* and *Protoiurus* species are strong and large-size scorpions that adults may exceed 100 mm's, contrarily *Calchas* and *Neocalchas* species are between 20-40 mm's long. To encounter with cave-dwelling populations of *Protoiurus* species are possible.

1.4.4 Euscorpiidae Family

Euscorpius is one of the well-studied genera among scorpions, belonging Euscorpiidae family. This genus highly dispersed in Southern Europe and Anatolia; and may be found in diverse habitats. According to up-to-date taxonomic research, Turkey has six valid Euscorpius species that are namely: *Euscorpius italicus*, *Euscorpius lycius*, *Euscorpius avcii*, *Euscorpius rahsenae*, *Euscorpius gocmeni* and *Euscorpius mingrelicus* (Figure 1.5f). Further, *E. mingrelicus* has six subspecies: *E. m. mingrelicus*, *E. m. ciliciensis*, *E. m. phrygius*, *E. m. ollivieri*, *E. m. legrandi* and *E. m. uludagensis*. (Tropea et al. 2014) The stinging cases from these small-sized scorpions are very uncommon and mild.

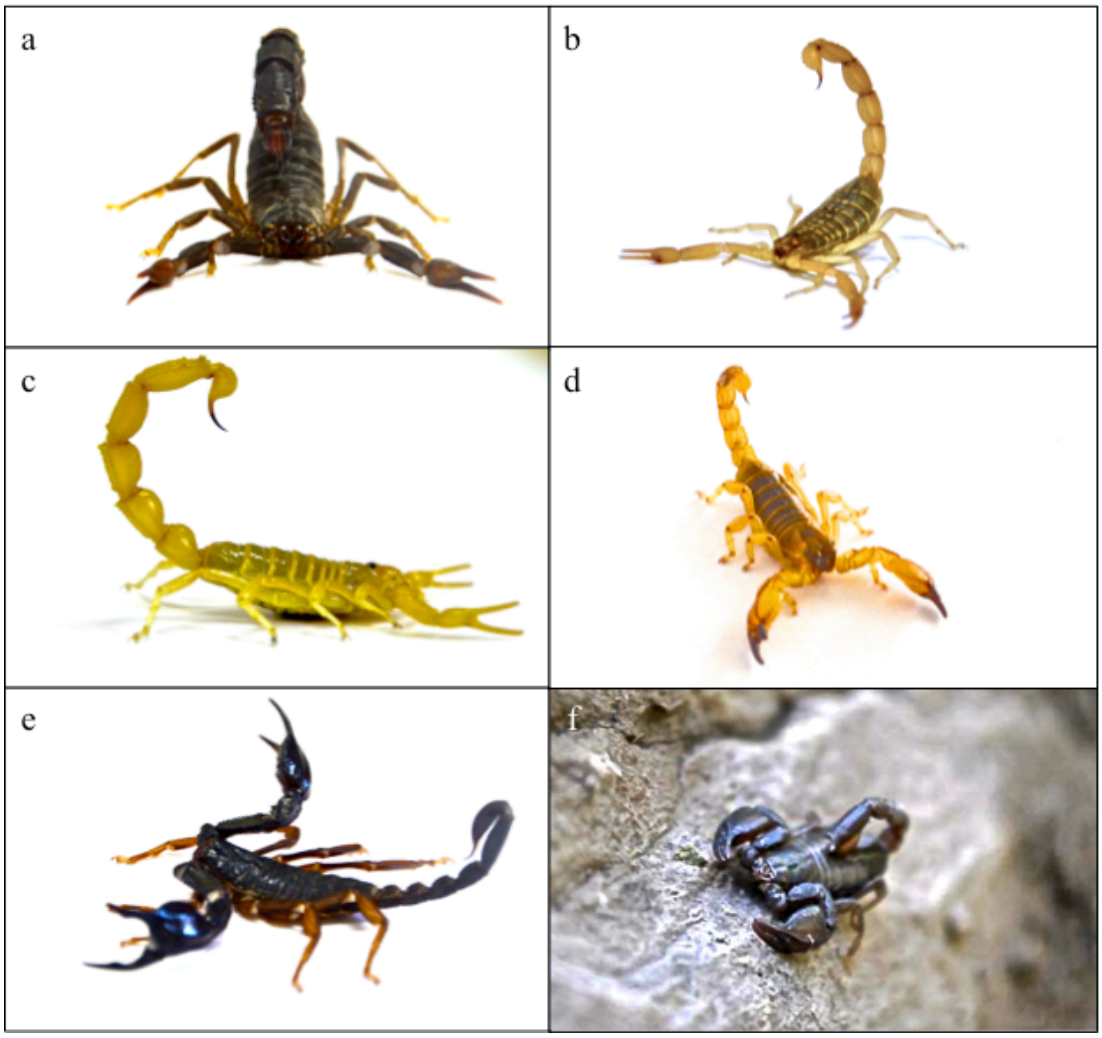

Figure 1.5 Selected species from Turkish scorpion fauna, from four different families. Some of medically important buthids of Turkey: *A. crassicauda* from Urfa province, Birecik district (a), *M. gibbosus* from Muğla province, Milas district (b) and *B. macrocentrus* from Urfa province, Birecik district (c). *S. maurus* from Urfa province, Birecik district (d). A large-sized iurid, *P. kraepelini*, from Antalya province, Alanya district (e). *E. mingrelicus* at its habitat in Ankara province, Kızılcahamam district (f).

1.4.5 Current State of Scorpion Venom Research in Turkey

Beside the rich and diverged scorpion fauna of Turkey, a small number of species' venom was characterized, currently. Until the last decade some initiative work on venom toxicity, anti-venom production and meta-studies based on clinical cases upon scorpion stings were achieved, to a certain extend. The Refik Saydam Hygiene Center (RSHC, now called Public Health Institute, PHI) is the main institution, where scorpion venom research took place and is supported by government (Caliskan 2014). The complete biochemical characterization of three buthid species was achieved by a venomics approach at last ten years. These are *A. crassicauda*, *B. macrocentrus* and *M. gibbosus*, chronologically. The venom characterization studies on *A. crassicauda* species identified eight unique long-chain peptides named Acra1 to Acra8 (Caliskan et al. 2006; Caliskan, Ergene, et al. 2013; Caliskan, Quintero-Hernández, et al. 2013; Caliskan 2014). The venom of *B. macrocentrus* also characterized and leads to discovery of a 65 amino acid mammalian specific Na^+ channel α-toxin, Bu1 (Caliskan et al. 2012). Three novel α-KTx type K^+ channel inhibitors named MegTx1, MegTx2 and MegTx3 were founded from the venom of scorpion *M. gibbosus* with the screening of the cDNA library generated from the expressed mRNAs in the venom gland. These toxins were observed to be belonging different α-KTx families α-KTx3.x, α-KTx9.x and α-KTx16.x, respectively (Diego-García et al. 2013). Also the venom protein profile of a number of individuals from Aegean population of *M. gibbosus* species were identified (Ozkan & Ciftci 2010).

1.5 The Genus *Leiurus*

1.5.1 Taxonomy and Distribution of *Leiurus* Genus

Leiurus is a widely distributed genus in North Africa, Middle East and Arabian peninsula; belonging Buthidae family. Ehrenberg firstly used the name *Leiurus* in 1828, corresponding to a subgenus of genus *Androctonus*. After then, in 1949, *Leiurus* was elevated to genus level by Vachon. *Leiurus* was known to be a

monotypic genus with a single species, *Leiurus quinquestriatus*. This genus has been no more considered to be monotypic, since 2002. Four new species were described through the year 2009 and they are *Leiurus jordanensis*, *Leiurus savanicola*, *Leiurus nasheri* and *Leiurus abdullahbayrami*. However, a recent comprehensive revision on *Leiurus* genus described four new species as well as *L. quinquestriatus hebraeus* and *L. quinquestriatus brachycentrus* subspecies elevated to species level. Also, the species *L. nasheri* was found to be synonym of *L. brachycentrus*. The current valid species of *Leiurus* genus are *L. quinquestriatus*, *L. jordanensis*, *L. abdullahbayrami*, *L. brachycentrus*, *L. macroctenus*, *L. haenggii*, *L. arabicus*, *L. heberti*, *L. savanicola* and *L. hebraeus*. *Leiurus* genus dispersed through the Libya, Sudan, Egypt, Israel, Jordan, Syria, Turkey, Lebanon, Saudi Arabia, Yemen, Qatar, United Arab Emirates and Oman, mainly. The *L. savanicola* of Cameroon is the only species found in equatorial climate (Lowe et al. 2014). Distribution of *Leiurus* species, except *L. savanicola*, can be seen on map in Figure 1.6. This genus is observed to show high coloration variations between different populations. Members of *Leiurus* genus are known to be health-threatening scorpions with medical importance. The type species of genus, *L. quinquestriatus* commonly called as “Deathstalker” that highlights its highly potent venom.

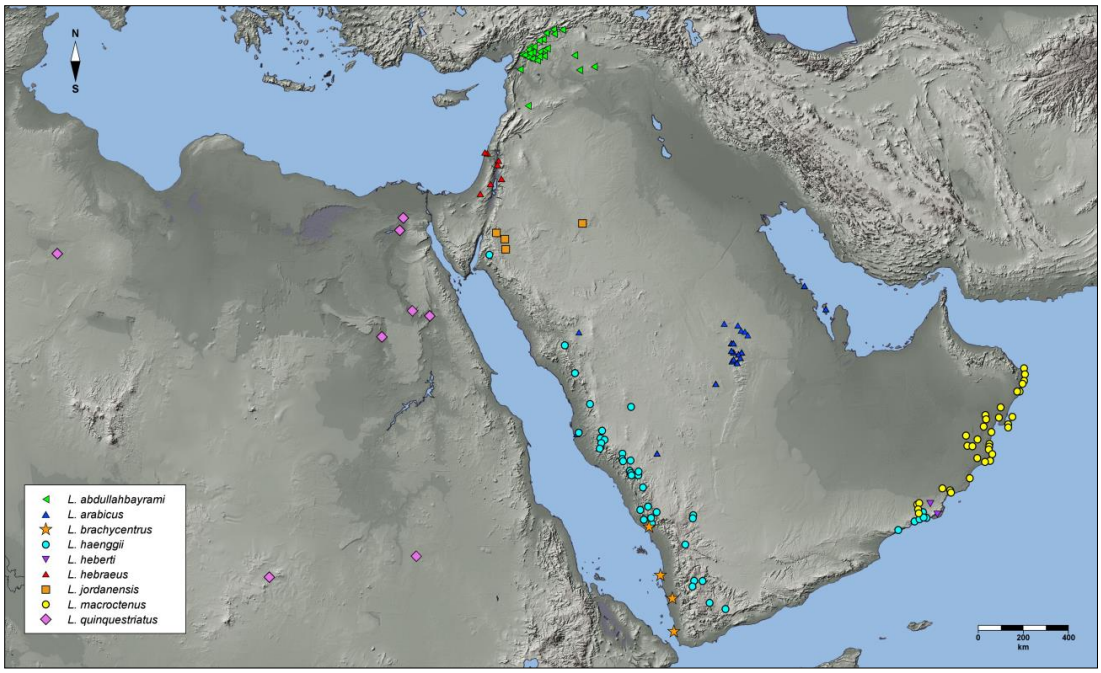

Figure 1.6 Distribution of *Leiurus* species, focusing on Arabian Peninsula, northeastern Africa and Middle East. (Lowe et al. 2014)

1.5.2 *Leiurus abdullahbayrami*

Leiurus abdullahbayrami is the only species of Turkey under the *Leiurus* genus. It is distributed in the west of Southeastern Anatolia and north of the Syria. *L. abdullahbayrami* population is much more denser at the western of the Euphrates basin, but limited number of specimens were recorded from the east side of the Euphrates river. The provinces that specimens had been recorded so far are Kahramanmaraş, Adıyaman, Gaziantep, Kilis, Hatay and Şanlıurfa. There were old unauthenticated records from Mardin and Diyarbakır provinces, yet they are still unproven despite the extensive field studies (Figure 1.6). First record of *Leiurus* from Turkey was at the year 1960 by Tulga, from Adıyaman province. However, until the year 2009, *Leiurus* from Turkey accepted as *L. quinquestriatus*. The detailed and comparative taxonomic characterization on the Turkish population of *Leiurus* genus was introduced many essential differences between Turkish population and type species *L. quinquestriatus*, which leaded up

to description of the new species *L. abdullahbayrami*. All Turkish and north Syrian *Leiurus* populations currently accepted as *L. abdullahbayrami* (Yağmur et al. 2009). Detailed view of *L. abdullahbayrami* can be seen in Figure 1.8.

Figure 1.7 Distribution of *L. abdullahbayrami* in Turkey. Gray area represents the probable distribution of species. The orange dashed field involves distribution according to unconfirmed records, which is still unclear and also was not confirmed by present study. Red arrow indicates the area, where specimens were collected. (www.mapbox.com)

Figure 1.8 *L. abdullahbayrami* scorpion in captivity, at defense position. Specimen is from Kilis province, Hasancalı Village.

The anatomical and pigmentation differences among three different *L. abdullahbayrami* populations of Turkey may lead to description of new species or subspecies at future. These differentiations were observed between Adıyaman-Gaziantep-Kilis, Şanlıurfa and Hatay populations (Figure 1.9). *L.*

abdullahbayrami generally prefer semi-arid steppe, rocky and basaltic habitats, but also recorded from chaparral areas (Yağmur et al. 2009).

Figure 1.9 Color variations in three different *L. abdullahbayrami* populations of Turkey. Hatay population (left), Şanlıurfa population (center) and Adıyaman-Gaziantep-Kilis population (right) could be seen. Scale is in millimeters (Yağmur et al. 2009).

1.5.3 Previous Studies on *L. abdullahbayrami* Venom

Ozkan et al. (2011) achieved the first study on *L. abdullahbayrami* venom. This

work presents the peptide profile of *L. abdullahbayrami* venom with Bis-Tris gradient gel electrophoresis and reports the LD_{50} value of crude venom on mice. They confirm the presence of both long-chain and short-chain peptide neurotoxins in venom as well as some low intensity protein bands. The LD_{50} value was found to be 0.19 mg/kg on mice, following the subcutaneous injection. The symptoms after venom injection were observed as aggressive behavior, agitation, hyperexcitability, squeaking, fighting, tachypnea, convulsions, weakness, paralysis, coma, and death. Death is primarily due to pulmonary edema subsequently followed by respiratory and cardiac arrest. The reported LD_{50} value is lower than that of *L. quinquestriatus*, whose LD_{50} value found to be 0.25 mg/kg on mice. This had been accepted to be the lowest exampled value among scorpion venoms, until the mentioned study. Thus, arguably, *L. abdullahbayrami* may be regarded as the scorpion species that produces the most potent venom against mammals. To confirm this statement; comparative, replicative and more extended research should be conducted.

1.6 Aim of the Study

The main aim of the study was to characterize peptide profile, as well as determine cytotoxicity and antimicrobial activity of *L. abdullahbayrami* venom. The protein/peptide content of crude venom was assessed and electrophoretic profiling was achieved by a comparative approach. The HPLC-SEC and HPLC-RP chromatograms were presented, where these methods were used primarily for fractionation of venomic peptides. The number and molecular weight of unique peptide masses were determined. A single mammalian cell line MCF-7 and a few selected pathogenic microbial species were used for bioactivity screening. Interestingly, a proliferative activity of crude venom on MCF-7 cells was observed. Crude venom was identified to have strong antibacterial potential against gram negative strains, mild antifungal activity and weak antibacterial

effect on gram positive bacteria.

This work is the first detailed biochemical characterization and activity screening of *L. abdullahbayrami* venom. These findings provide preliminary data for more detailed future studies. Further research on *L. abdullahbayrami* venom may lead to discovery of novel Na^+, K^+, Ca^{2+} and Cl^- ion channel inhibitor peptide neurotoxins as well as peptides with antimicrobial, immunomodulatory and hemolytic activities. Moreover, the electrophoretic peptide and protein profile and determined peptide masses, which are presented, may be used as a taxonomic tool for differentiation and determination of scorpion species.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

2.1.1 Scorpion Specimens

L. abdullahbayrami scorpions were collected from semi-arid steppe area in Sinanköy village, Gaziantep province, Southeastern Turkey. The coordinates are: 37°2’16”N 37°35’58”E.

2.1.2 Chemicals and Reagents

2.1.2.1 Protein Content Determination

Bio-Rad Quick Start™ Bradford Protein Assay with gamma-globulin as standard protein (USA) was used for protein content determination of the crude venom and venom fractions.

2.1.2.2 Electrophoretic Analyses

Acrylamide solution 40% for molecular biology, Bisacrylamide for molecular biology, Tris ($C_4H_{11}NO_3$) for molecular biology, SDS ($C_{12}H_{25}NaO_4S$) for molecular biology, Ammonium persulfate ($(NH_4)_2S_2O_8$) for molecular biology,

TEMED ($C_6H_{16}N_2$), Urea crystalline (NH_2CONH_2) Molecular Biology grade and Glycine (H_2NCH_2COOH) for molecular biology were purchased from Applichem (Germany). Tricine ($C_6H_{13}NO_5$) powder, 2-mercaptoethanol (C_2H_6OS), Laemmli Sample Buffer and Tricine Sample Buffer were obtained from Bio-Rad (CA, USA). These were used in the polymerization of polyacrylamide gels, sample preparation and running of the experiments.

Agilent Protein 80 kit (USA) was used for MCE analysis of the crude venom.

Silver nitrate ($AgNO_3$) for molecular biology, Formaldehyde - Solution 37% technical grade, Sodium thiosulfate ($Na_2S_2O_3$) anhydrous, Potassium carbonate (K_2CO_3) technical grade, Methanol (CH_3OH) HPLC grade, Ethanol (CH_3CH_2OH) absolute for molecular biology, Acetic acid (CH_3COOH) 100% for molecular biology purchased from Applichem (Germany) used in preparation of Silver Staining solutions for staining of Tris-Glycine and Tris-Tricine gels.

2.1.2.3 HPLC Fractionation

Tosoh Bioscience TSKgel® G2000SW size exclusion column, 7.5 mm × 600 mm 12.5 nm pore size (Japan) and Vydac® 218TP54 C18 reversed- phase column, 4.6 mm × 250 mm 300 Å pore size (USA) were used in the HPLC fractionation of the venom. Applichem Acetonitrile HPLC grade (Germany) and Applichem Trifluoroacetic acid Spectroscopy grade (Germany) were used in preparation of mobile phases.

2.1.2.4 Mass Spectrometry

Agilent Technologies ZORBAX Eclipse XDB C18 column, 4.6 mm × 150 mm 5 µm pore size (USA) was used in Mass Spectrometry analysis of the venom fractions. Applichem Acetonitrile for LC-MS and Applichem Formic acid 98-100% Technical grade (Germany) were used in preparation of mobile phase.

2.1.2.5 Cell Culture

Dulbecco's Modified Eagle's Medium (DMEM) (Table A.1), Fetal Bovine Serum (FBS), Penicillin-Streptomycin (Pen-Strep) and Trypsin-EDTA were purchased from Lonza (Switzerland). Sterile 1X Phosphate Buffered Saline (PBS) without calcium and magnesium for cell culture (pH: 7.4) was supplied from Gibco® (Invitrogen, USA) (Table A.5). Cell Signaling Technology XTT Cell Viability Kit (USA) was used for the investigation of cytotoxic/proliferative effect of the crude venom. Etoposide obtained from Sigma Aldrich (USA) was used as positive control with a single dose of 60 μM. MCF-7 human epithelial ER (Estrogen Receptor) positive breast adenocarcinoma cell line was obtained from Assoc. Prof. Dr. Sreeparna Banerjee, Department of Biological Sciences, Middle East Technical University.

2.1.2.6 Microbial Culture

Listeria monocytogenes, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Candida krusei* and *Candida albicans* strains were provided from the Laboratory of Microbiology, Medical Faculty, Kırıkkale University. Mueller Hinton broth, Mueller Hinton agar and Sabouraud dextrose-agar media purchased from Sigma Aldrich (USA). Antibiotic discs AMC-30, E-15, RA-5 and TE-30 containing 30 μg amoxicillin with clavulanic acid, 15 μg erythromycin, 5 μg rifampin and 30 μg tetracycline, respectively, obtained from BD (USA).

2.2 Methods

2.2.1 Maintenance of Scorpion Specimens

2.2.1.1 Field Studies

L. abdullahbayrami scorpions were collected from semi-arid steppe regions of Gaziantep province, Şehitkamil district, Sinanköy village at coordinates: 37°2'16"N 37°35'58"E. Field studies were conducted during summer season at moonless nights as well as daytime. In night field studies, scorpions were collected under UV light (F8T5/BLB lamp), which illuminates cuticle of scorpions greenish. In daytime field studies, scorpions were obtained by removing rocks. Scorpions collected with forceps and transferred to plastic boxes. Three female and six male adults were collected at total.

2.2.1.2 Scorpion Feeding

Scorpion specimens were fed in plastic boxes and the bottom of the box was covered with cocopeat as substrate. Scorpions were fed weekly with mealworms, cockroaches and grasshoppers. The entrance of book lungs were cleaned with medical grade hydrogen peroxide solution and boxes treated with antifungal agents monthly to prevent fungal infections. Boxes humidified with water periodically.

2.2.1.3 Venom Milking

Before venom collection, scorpions were anesthetized with CO_2. Scorpion fixed on a polyethylene panel by rubbers. The telson of the scorpion placed in a polypropylene PCR tube (Greiner, Germany). Venom milking was conducted by electrical stimulation of the telson with 15 V, low current DC power supply. The crude venom from all specimens pooled and walls of the tube washed with sterile ddH_2O to collect remaining. The venom solution centrifuged at 15.000 g for 15

minutes at 4°C. Soluble fraction of the crude venom transferred into a previously weighed 2 mL polypropylene tube and lyophilized subsequently. The tube was weighed again to calculate dry weight of the crude venom. Tube sealed with Parafilm® (Sigma Aldrich, USA) and stored at -80°C until use.

2.2.2 Protein Content Determination

Protein content of crude venom and venom fractions were determined with Bio-Rad Quick Start™ Bradford Protein Assay, in 96-well microplate format as it is described in the user's manual (BioRad). The plate was read at 595 nm absorbance wavelength with SpectraMax 340PC plate reader (Molecular Devices, USA). The standard curve was generated and protein content in the sample wells calculated by the software SoftMax® Pro 6 (Molecular Devices, USA) automatically.

2.2.3 Electrophoretic Analyses

Bio-Rad Mini Protean tetra cell electrophoresis system (USA) and Bio-Rad PowerPac™ universal power supply (USA) was used for Tris-Glycine SDS-PAGE and Tris-Tricine SDS-PAGE analyses. For MCE analysis, Agilent Bioanalyzer (USA) was used.

2.2.3.1 Tris-Glycine SDS-PAGE

The gel casting apparatus was assembled as it is described in user's manual. Two gels: 10% separating and 4% stacking were used in this method. The 10% gel solution prepared from stock solutions according to Table B.1 (Appendix B) and poured between glasses up to 0.5 cm lower of the position of the combs. Isopropanol was added on the gel to provide a flat surface. After 30 minutes of polymerization, the isopropanol was removed. The 4% gel solution was prepared with respect to Table B.1 (Appendix B). The solution was poured between glasses until let it flow. The 10-well comb placed subsequently and gel polymerized for 45 minutes. Comb was removed gently and gel holding glasses disassembled from

casting apparatus and placed in electrophoresis cell as it is described in user's manual. The cell was placed in electrophoresis tank. Inner and outer chambers were fulfilled with 1X Tris-Glycine Gel Running Buffer (Appendix B). 5 µl of sample containing 5 µg of protein was mixed with Bio-Rad Laemmli Sample Buffer (USA) containing 5% 2-mercaptoethanol (1:1) in PCR tubes (Greiner, Germany) and 10 µl resulting sample mix denatured at 95^{o}C for 5 minutes with pre-heated heat block. The tubes were centrifuged by micro spinner for 10 seconds and loaded in wells. The running conditions were set as constant 25 mA/gel.

2.2.3.2 Tris-Tricine SDS-PAGE

The Tris-Tricine SDS-PAGE protocol is derived from the protocol of (Schägger 2006). The gel casting apparatus was assembled as it is described in the user's manual. Three gel layers with different percentages and content were used and those are 16% separating gel with 6 M urea, 10% spacer gel and 4% stacking gel from bottom to top, respectively. The gel casting glass was marked from top to bottom to determine gel positions. Stacking gel ends 0.5 cm lower of the end position of the combs, spacer gel covers 1 cm area between the stacking and separating gels, where the rest of the bottom covered by the separating gel. 16% separating gel solution with 6 M urea was prepared from stock solutions according to Table B.2 (Appendix B) and subsequently poured according to previously marked levels between glasses before polymerization. The top of the gel was sealed with isopropanol to provide a flat horizontal surface to separating gel. After 30 minutes, which is enough for the complete polymerization, the isopropanol was removed. The 10% spacer gel solution was prepared according to Table B.2 (Appendix B) and was rapidly poured over polymerized 16% gel. Again, isopropanol was added to flatten the surface. After 30 minutes of incubation, isopropanol removed. The 4% stacking gel solution was prepared as described in Table B.2 (Appendix B) and poured between glasses until it flows. The 10-well comb placed and pushed inside the stacking gel. After 45 minutes of

incubation, comb was removed and gel holding glass pair was separated from the casting apparatus. The gel between glasses was placed into electrophoresis cell and then cell placed into electrophoresis tank. The inner chamber of the cell was filled completely with cathode buffer (Appendix B) and the outer chamber was filled with anode buffer (Appendix B). 5 µl of sample containing 5 µg of protein mixed with Bio-Rad Tricine Sample Buffer (USA) containing 5% 2-mercaptoethanol (1:1) in PCR tubes (Greiner, Germany) and 10 µl resulting sample mix denatured at 95^{o}C for 5 minutes with pre-heated heat block. The tubes were centrifuged by micro spinner for 10 seconds and loaded in wells. The running conditions were set as 15 mA/gel until the samples pass through the stacking gel and elevated to 35 mA/gel constantly to the end of the run.

2.2.3.3 Silver Staining

Tris-Glycine SDS-PAGE and Tris-Tricine SDS-PAGE gels were subjected to silver staining following their run to observe protein bands. After electrophoresis, gels removed from casting glasses and stacking gel was chopped. Resulting gel was placed into a plastic tray filled with gel fixing solution (Appendix B) and was incubated overnight at 4^{o}C. Gel fixing solution removed and gel washed three times with 50% ethanol solution (v/v) (Appendix B) for 20 minutes each on racking plate. 50% ethanol solution was poured and gel treated with pre-treatment solution (Appendix B) for 1 minutes. Pre-treatment solution was removed and gel rinsed three times with dH_2O (obtained from MilliQ® Integral Water Purification System, Millipore) for 20 seconds each. Silver nitrate solution (Appendix B) was added on gel and incubated for 20 minutes on racking plate. Silver nitrate solution was poured and gel rinsed three times with dH_2O for 20 seconds each. Developing solution (Appendix B) was added and gel observed for the appearance of bends. Roughly after 1-2 minutes bands was observed to develop. Gel was immediately rinsed 3 times with dH_2O and placed into stop solution (Appendix B) to prevent over staining. Gel images were taken by Vilber Lourmat gel documentation system (France).

2.2.3.4 Microfluidic Capillary Electrophoresis

Agilent Protein 80 kit (USA) was used in MCE analysis of the crude venom, as it is described in the user's manual (Agilent Technologies). 0.1 μg of protein loaded per well.

2.2.4 HPLC Fractionation

Varian Prostar HPLC system (USA) was used in the chromatographic analysis and fractionation of the venom.

2.2.4.1 Size Exclusion Chromatography

L. abdullahbayrami crude venom in concentration of 4 mg/mL and total volume of 50 μL of reconstituted in SEC running buffer (Appendix B) was applied to Tosoh Bioscience TSKgel® G2000SW size exclusion column, eluted with constant flow rate of 0.5 mL/minute with SEC running buffer and run for 60 minutes. The absorbance was read at 210 nm. The peaks on the chromatogram were observed and fractions obtained manually in polypropylene tubes. Peptide and protein fractions were collected, lyophilized and frozen at -80^{o}C to use in further fractionations and activity assays.

2.2.4.2 Reversed Phase Chromatography

Peptide fraction previously taken from size exclusion chromatography was dissolved in Solution A for reversed phase chromatography (Appendix B) yielding a concentration of 30 μg/mL at total volume of 50 μL. This peptide fraction was loaded to Vydac® (USA) 218TP54 C18 reversed phase column. A linear gradient from solution A for reversed phase chromatography to 60% solution B for reversed phase chromatography (Appendix B) with a flow rate of 0.7 mL/minute applied for 90 minutes. The absorbance was read at 210 nm. Fractionated peptides were collected manually according to observed peaks on chromatogram in polypropylene tubes. The acetonitrile content resulting from

solution B for reversed phase chromatography was vaporized by rotary evaporation and remaining water again lyophilized. Fractions were stored at -80^{o}C until first use.

2.2.5 Mass Spectrometry

Agilent 1200 HPLC in-line with Agilent LC-TOF/MS 6530 (USA) mass spectrometer unit (UNAM, Bilkent University) was used in the determination of peptide masses in venom. Previously collected peptide fraction with size exclusion chromatography reconstituted in buffer A for LC/MS TOF (Appendix B) yielding 30 µl/mL concentration in volume of 50 µL. Peptide mix was applied Agilent Technologies ZORBAX Eclipse XDB C18 reversed phase column. Peptides eluted by a linear gradient from buffer A for LC/MS TOF to 60% buffer B for LC/MS TOF (Appendix B) with 0.7 mL/minute flow rate and over 90 minutes. The system was coupled with an ESI unit, which provides ionization. Mass detection was achieved by TOF module, which was set to 2000 V, 100-3200 m/z ratio and positive ion mode. Analysis of the data performed manually on Agilent MassHunter Workstation Qualitative Analysis (USA) platform.

2.2.6 Cell Culture

2.2.6.1 Cell Culture Conditions

MCF-7 cells were maintained in DMEM (Appendix A) supplemented with 10% (v/v) FBS and 1% (v/v) Pen-Strep (This medium referred as complete medium). Cells were incubated at 37^{o}C temperature and 5% (v/v) CO_2 with humidified Thermo Scientific (USA) incubator. Cells were cultured in T-25, T-75 and T-175 sterile tissue culture flasks (Greiner, Germany).

2.2.6.2 Subculturing

Cells were subcultured, when the cell confluency reaches 80% in tissue culture flask. The medium was aspirated and cells were washed twice with 1X PBS. The 1X Trypsin-EDTA solution was added to the flask. Cells were observed under light microscope to see detachment process. When cells become more globular compared to their standard morphology, the Trypsin-EDTA was removed to prevent excess proteolytic activity to cell surface proteins. Cells were incubated at 37^{o}C for 5 minutes, and then complete medium was added to flask to resuspend cells. Cell suspension was divided equally into new flasks.

2.2.6.3 Cell Stocking

A 80% confluent T-75 full of detached cells were resuspended in 5 mL of complete medium and transferred into a 15 mL falcon tube (Greiner, Germany) and centrifuged (Hermle, Germany) at 250 g for 5 minutes. The supernatant was aspirated and pellet was resuspended in 3 mL of ice-cold complete medium supplemented with 10% (v/v) DMSO (Applichem, Germany). 1 mL aliquots were transferred into cryovials (Greiner, Germany). The vials were frozen in -80^{o}C refrigerator, overnight, and then transferred into liquid nitrogen for long term storage.

2.2.6.4 Cell Thawing

Previously prepared frozen cell stock solution was placed into water bath at 37^{o}C to let it thaw rapidly. After complete thawing of the stock solution, 4 mL of complete medium was added on it and transferred in a 15 mL falcon tube. Cells were centrifuged at 250 g for 5 minutes and supernatant was aspirated to remove DMSO. The remaining pellet was resuspended with 5 mL of complete medium and transferred into T-25 flask as starter culture.

2.2.6.5 Cell Viability Assay

Crude venom's effect on cell viability was determined with Cell Signaling Technology XTT Cell Viability Kit (USA). MCF-7 cells were suspended in complete medium and were seeded on clear and flat bottom sterile 96-well plate (Greiner, Germany) as $2x10^4$ cells/100 µL per well, 24 hours prior to treatment to provide enough cell adhesion and confluency. A single dose of crude venom, 200 µg/mL and 60 µM of Etoposide solutions were prepared in FBS-free DMEM (from now on, this formulation referred as serum-free medium) to exclude neutralizing effect of serum proteins on venom components. The complete media of the previously seeded cells were aspirated. 200 µg/mL crude venom, 60 µM Etoposide as positive control and only serum-free medium as negative control were applied to cells at total volume of 100 µL. Treated cells were incubated at previously mentioned cell culture conditions for 24 hours and 48 hours. After the completion of the incubation, XTT working solution was prepared freshly with mixing electron coupling solution and XTT reagent in 1:50 ratio (v/v). 50 µL of XTT working solution was added to each well without aspirating treatment mediums and cells were incubated for another 4 hours at cell culture conditions. Resulting colorimetric changes were read with SpectraMax 340PC plate reader at two wavelengths: 490 nm and 650 nm. The background absorbance due to any cell debris was excluded from exact reading by subtracting absorbance value at 650 nm from 490 nm. The 100 µL serum-free medium with 50 µL of XTT working solution was used as blank. All experiments were conducted in triplicates.

2.2.7 Microbial Culture

2.2.7.1 Culturing of Bacterial Species and Antibacterial Activity Screening

Mueller Hinton broth media (Appendix A) and Mueller Hinton agar media (Appendix A) were prepared by dissolving 23 g and 38 g in 1 L dH_2O, respectively. For sterilization, media were autoclaved at 121^oC for 15 minutes. Bacterial species *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* were activated via inoculation of a loop from glycerol stock to Mueller Hinton broth media, then incubated at 37^oC in shaking incubator for 18 hours. Then 200 µL of fresh inoculum (10^7 – 10^8 cells/mL) was spread to plates containing Mueller Hinton agar media. 40 µL *L. abdullahbayrami* crude venom (20 mg/mL) soaked disks, as well as antibiotic discs placed at the center of the agar plates. Plates were incubated for 18-24 hours at 37^oC. After all, bacterial growth inhibition was measured with respect to diameter of inhibitory zone in terms of millimeters. 1X PBS soaked discs were used as negative control.

2.2.7.2 Culturing of Fungal Species and Antifungal Activity Screening

Sabouraud dextrose agar (Appendix A) prepared by dissolving 65 g of media powder in 1 L dH_2O and followed by autoclave sterilization at 121^oC for 15 minutes. Two pathogenic fungal species, *Candida krusei* and *Candida albicans*, (0.5-2.5 x 10^6 cells/mL) were cultivated on Sabouraud dextrose agar plates. 40 µL *L. abdullahbayrami* crude venom (20 mg/mL) soaked disks, as well as antibiotic discs placed at the center of the agar plates. Plates were incubated for 36-48 hours at 25^oC and growth inhibition was measured with respect to diameter of inhibitory zone in terms of millimeters. 1X PBS soaked discs were used as negative control.

2.2.8 Statistical Analysis

Statistical analysis was performed for the cell viability assay. The data represented as mean and error bars represent SD ($n = 3$). Stars (*, **, ***) highlight

statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. The analysis was performed on GraphPad Prism Software (GraphPad Software Inc., USA) and One-way ANOVA test followed by Tukey's multiple comparison test were applied.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Field Observations

During the field studies some observations on the habitat selection and behavior of *L. abdullahbayrami* were observed. *L. abdullahbayrami* scorpions were seen to active through April to October months of a year. In daytime, they are encountered under rocks; while nighttime they are observed to be highly active. They prefer to live in habitats with low vegetation. *L. abdullahbayrami* generally co-appears with the *S. maurus* and *M. nigrocinctus* species. High population of crickets and grasshoppers are observed in the places, where *L. abdullahbayrami* appears highlighting its probable natural diet. Contrary to many scorpion species, which tend to escape upon capturing, *L. abdullahbayrami* keeps its defensive position and resist. This aggressive behavior of the species was also observed during feeding and venom milking.

3.2 Protein Content and Protein Profile of *L. abdullahbayrami* Crude Venom

The protein and peptide content of *L. abdullahbayrami* venom was determined and found to be 54% of the dry weight of crude venom, approximately. This value is relatively close to that of a member of the same genus, *L. quinquestriatus*, which was reported as 65% (Chicchi et al. 1988). More than 50% of the venom

content comprises peptides and proteins, which is an expected result and indicates that major part of the crude venom is composed of toxins in peptidic structure.

Electrophoretic profiling of *L. abdullahbayrami* crude venom was achieved by three different electrophoretic methods, Tris-Glycine SDS-PAGE, Tris-Tricine SDS-PAGE and MCE. The Tris-Glycine SDS-PAGE uses the advantage of well separation of high MW proteins. Thus, this technique is mainly used for the observation of protein content of the crude venom. The high MW proteins of the scorpion venoms involve phospholipase and hyaluronidase-family enzymes. Tris-Glycine SDS-PAGE profile of the *L. abdullahbayrami* crude venom yields an intense 70 kDa band and weak bands between 20-70 kDa's (Figure 3.1 a). The protein appearing at 70-kDa may be an abundant enzyme of the venom content. Normally, scorpion venom profiles are much more enriched in protein bands. However, *L. abdullahbayrami* crude venom lacks these bands. This observation was confirmed by previous studies (Ozkan et al. 2011; Caliskan 2014), so this may be thought as a characteristic feature of *L. abdullahbayrami*. Other than the protein bands, a broad peptide band around 10 kDa can be seen. As expected, the resolving capacity of Tris-Glycine SDS-PAGE is not enough for the separation of individual peptide bands.

To focus on the peptide profile of *L. abdullahbayrami* venom, we have chosen the Tris-Tricine SDS-PAGE method that offers a better resolution of proteins smaller than 10 kDa. As recommended for peptides with complex and tight structure, based on the knowledge of cysteine-rich nature of peptide toxins, the resolving gel was prepared with 6 M urea to facilitate on gel denaturation. Also we used three layers of gels 4%, 10% and 16% to obtain a better resolution (Schägger 2006). Proteins of crude venom with higher MWs than 40 kDa were observed to be stacked at the joint between 10% and 16% gels and they could not pass through the separating gel. The stacked high MW proteins could be seen as an intense band at the top of the gel (Figure 3.1 b). The Tris-Tricine gel, as we thought, provides a better resolution for the peptides and resolves the 10 kDa band of Tris-

Glycine SDS-PAGE to 8 kDa and 10 kDa bands (Figure 3.1 b). However, still we could not achieve to observe expected long-chain and short-chain peptide toxins at the corresponding MWs of 3 to 7 kDa. In spite of many optimization experiments including changing the reducing agent concentration, switching between different reducing agents such as beta mercaptoethanol to DTT and using various denaturation conditions at sample preparation step prior to loading; we were not able to resolve peptide bands. It is probably because of intermolecular cross-linking issue, due to high cysteine-bridge content of venomic peptides. The possibility of a problem due to resolving capacity of separating gel was excluded, since we observed a remarkable resolution of peptide bands of the protein ladder (Figure C.1). Clearly, the 16% separating gel was enough to resolve 3.4 and 5 kDa peptide masses.

The MCE analysis of the crude venom was conducted by Protein 80 Kit and Agilent Bioanalyzer device. This is the first reported use of this kit in a venomic characterization study in literature. At first look, MCE technique obviously requires about 50 times less sample compared to Tris-Glycine and Tris-Tricine methods. Moreover, it provides a better resolution for both peptides and proteins, simultaneously. The expected individual peptide bands appear at 6 and 7 kDa's as well as the intense 70 kDa band of Tris-Glycine SDS-PAGE divided into separate bands of 71, 78 and 83 kDa bands (Figure 3.1 c). 78 kDa band was identified to be most intense one, probably comprising major content of previous 70 kDa band in Tris-Glycine gel. The short-chain neurotoxins were still not observable, since their MW overlap to system peak of device. Nevertheless, the intensity and broadness of the system peak increased with respect to its normal state, which indicates the presence of short-chain peptide toxins at the MW of 3-4 kDa's. MCE's clear resolution advantage over other electrophoretic techniques that were used may be seen. However, Protein 80 Kit originally designed for the examination of proteins at the MW range of 5-80 kDa's. The system peak interference makes the profiling of short-chain neurotoxins indeed hard, yet MCE could be a promising alternative

to Tris-Glycine and Tris-Tricine techniques for the observation of venomic peptides/proteins larger than 5 kDa.

The electrophoretic profiling of *L. abdullahbayrami* crude venom shows the presence of high MW proteins as well as peptides smaller than 15 kDa. The major peptidic biomolecules of the crude venom consist of peptides with MW lower than 10 kDa, which are expected to be peptide neurotoxins. The obvious advantages and significant limitations of MCE to other electrophoretic methods are also highlighted.

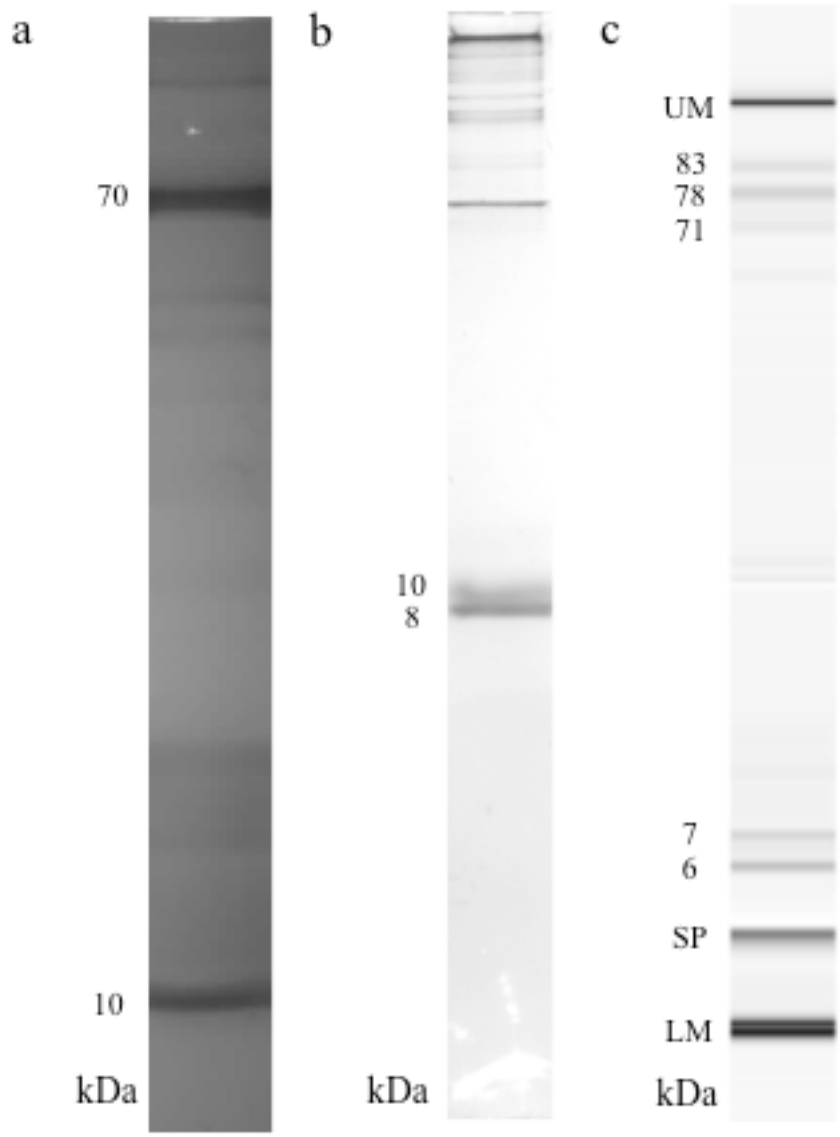

Figure 3.1 Electrophoretic profile of *L. abdullahbayrami* crude venom with (a) Tris-Glycine SDS-PAGE, (b) Tris-Tricine SDS-PAGE and (c) MCE. UM, LM and SP stand for upper marker, lower marker and system peak, respectively.

3.3 Size Exclusion Chromatography

Since the main focus of the study is peptidomic characterization of the *L. abdullahbayrami* venom, proteins with molecular masses lower than 1 kDa and higher than 15 kDa should be excluded. To achieve this, size exclusion chromatography (a.k.a. gel filtration chromatography) was selected. Briefly, working principle of size exclusion columns is to fractionate components of a mixture according to their sizes. A gel matrix is the stationary phase of the column and provides pores. The larger masses that cannot enter the pores migrate faster in the column; while smaller masses stack between the pores of the matrix, which results in a delayed elution time. Therefore, proteins with high MWs are expected to elute firstly from the column, than the peptide masses and lastly small organic molecules and ions.

To predict MWs of the venomic components according to retention times, an optimization experiment with standard proteins of known MWs was initially conducted. Carbonic Anhydrase, Ribonuclease A and Aprotinin were mixed in equal concentrations and loaded to gel filtration column and three major peaks in chromatogram from those masses recorded. The logarithms of the MWs of standard proteins calculated and a standard curve with respect to theirs' retention times generated (Table 3.1, Figure 3.2). By using the equation of the standard curve, we were able to generate a RT versus MW scale. This knowledge, indeed, facilitates the collection of exact peptide and protein fractions from SEC.

Table 3.1 Molecular weights, calculated logarithm of molecular weights and retention times at HPLC-SEC of standard proteins.

Standard Protein	Molecular Weight (Da)	Log [MW]	Retention Time (min)
Carbonic Anhydrase	29000	4.46	28.21
Ribonuclease A	13700	4.14	32.52
Aprotinin	6500	3.81	35.66

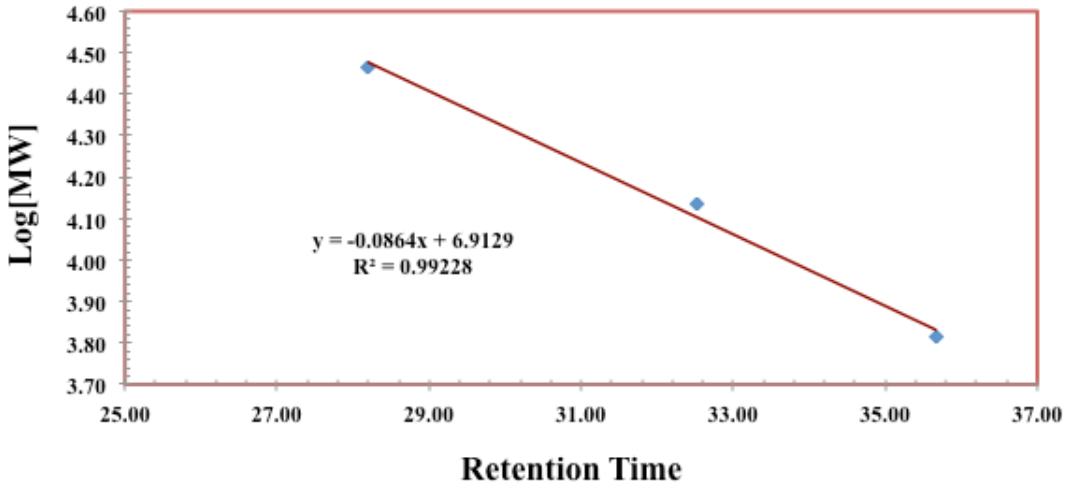

Figure 3.2 The standard curve of retention times versus logarithm of standard proteins' MWs, generated by the run in HPLC-SEC.

The run of the crude venom in HPLC-SEC yields to an initial protein peak eluted between 25-30 minutes (Figure 3.3, indicated as "1"). The top of the peak

indicates a 36 kDa protein according to expected MW scale. RTs between 30-40 minutes, a major peptide peak with two shoulders, demonstrate the presence of multiple peptides obtained (Figure 3.3, indicated as "2"). Two minor peaks were also recorded after 45th minutes of the run. Predicted protein and peptide fractions were collected for SDS-PAGE confirmation and further experiments, where last two peaks were discarded since those are out of our focus.

The obtained protein (1) and peptide (2) fractions were loaded to Tris-Glycine and Tris-Tricine gels, respectively. The sharp protein peak that was expected have a MW of 36 kDa was appeared as 70 kDa band in the gel (Figure 3.4, left panel). This phenomenon may be due to highly globular structure of the claimed protein, which may tend to behave as a smaller mass in the SEC column. On the other hand, as predicted, peptide fraction yields to a single peptide band of 9 kDa (Figure 3.4, right panel). Again the cross-linking of venomic neurotoxins prevents fully separation of the short-chain and long-chain peptides.

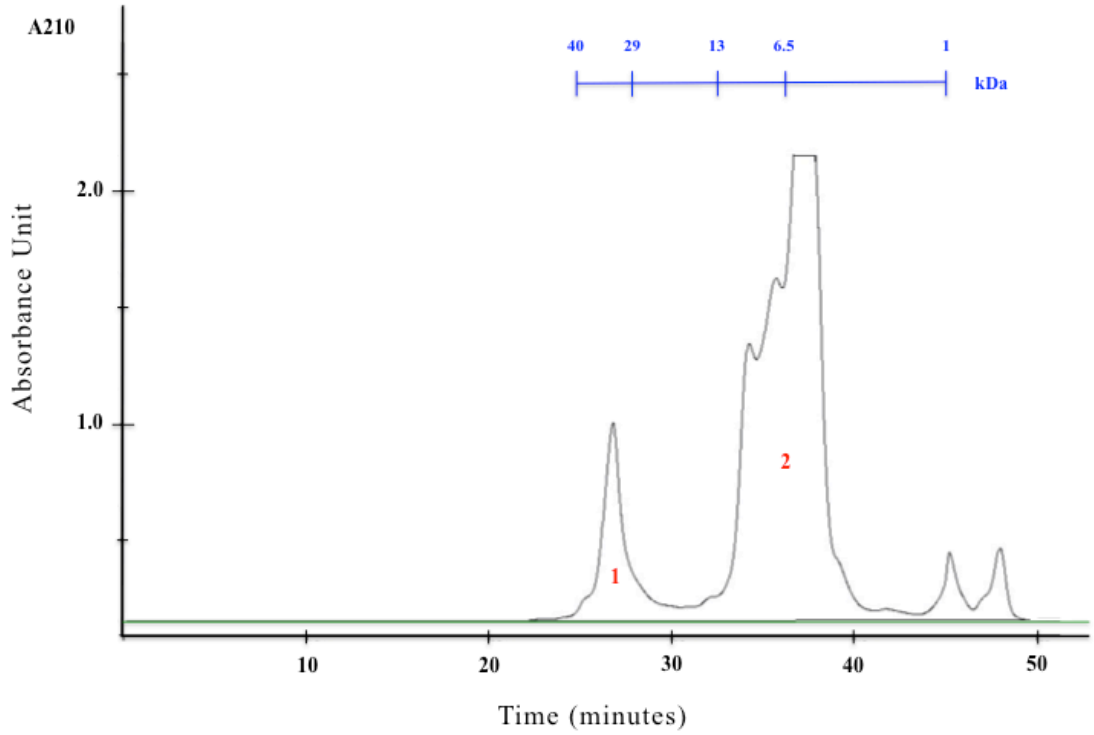

Figure 3.3 HPLC-SEC chromatogram of *L. abdullahbayrami* crude venom. Blue scale represents the estimated molecular weights versus retention time. The protein fraction and the peptide fraction named as 1 and 2, respectively.

The SDS-PAGE results support the proper fractionation of the protein and peptide fractions by HPLC-SEC. The peptide fractions collected from various HPLC-SEC runs were used for further analyses and experiments such as HPLC-RP and LC/MS TOF.

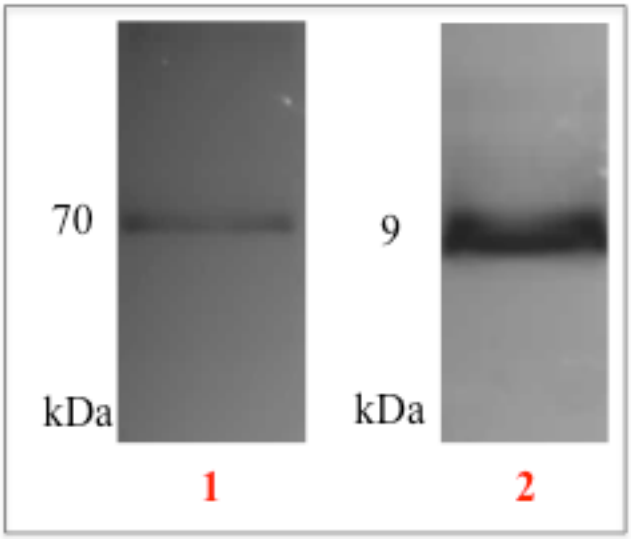

Figure 3.4 Tris-Glycine (left) and Tris-Tricine SDS-PAGE profiles of collected protein (1) and peptide (2) fractions from HPLC-SEC.

3.4 Reversed Phase Chromatography

As it has been mentioned before, bimodal distribution of short-chain and long-chain peptide toxins of scorpion venoms makes it impossible to further fractionate peptide mixture with SEC. Reversed phase chromatography is an invaluable tool for the separation of peptides with same MWs, since it separates molecules according to their physicochemical properties rather than sizes. In detail, the C-18 matrix of the reversed phase column provides binding surfaces for molecules as stationary phase, where molecules eluted serially by an increasing gradient of organic solvent as mobile phase. In principle, more hydrophilic molecules disengaged from stationary phase at lower concentrations of the gradient and more hydrophobic ones leave more lately. Therefore, by HPLC-RP, we were able to fractionate overall peptide mass into individual or small groups of peptides.

Peptide fraction collected by SEC loaded to reversed phase column and over 90 minutes run with an increasing ACN gradient, the HPLC-RP chromatogram of *L. abdullahbayrami* venom peptides was generated (Figure 3.5). 16 major peptide peaks were observed and largest 4 of them were selected and named from 1 to 4.

The collected peptide peaks were loaded to Tris-Tricine SDS-PAGE for confirmation. At total, peptides masses from 7 to 15 kDa were observed (Figure 3.6). 1st peptide fraction was understood to consist of 4 peptides with molecular weights of 7, 8, 10 and 12 kDa's. The 2nd peptide fraction having the sharpest peak in HPLC-RP chromatogram yields to a weak 15 kDa band, indicating the presence of larger peptides in *L. abdullahbayrami* venom over 10 kDa. 3rd peptide fraction again consist of larger peptides at MWs of 11 and 13 kDa's. Interestingly, 4th peptide fraction has a 9 kDa intense peptide band, which were previously observed in SDS-PAGE experiments on SEC peptide fraction.

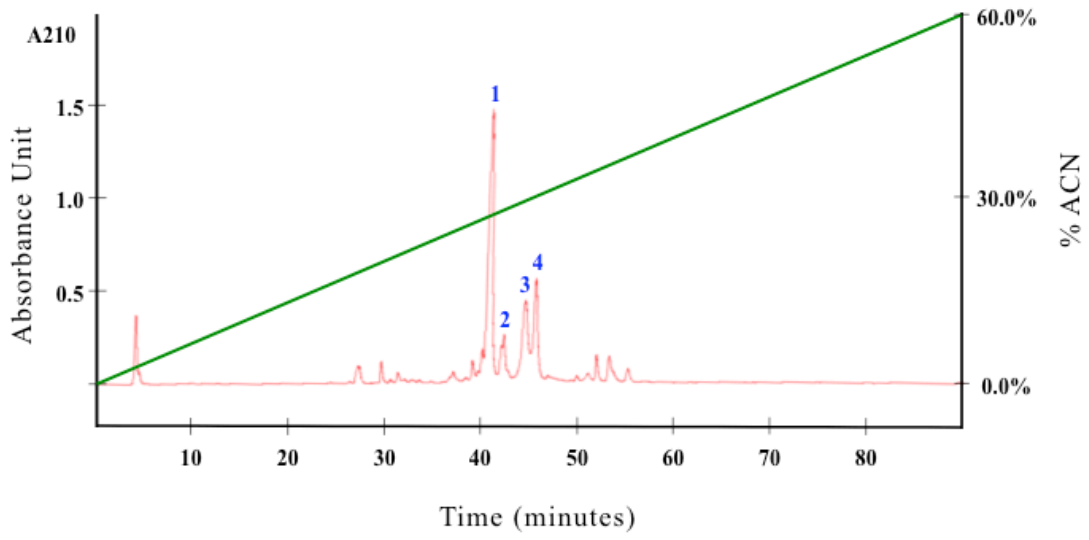

Figure 3.5 HPLC-RP chromatogram of *L. abdullahbayrami* venom peptide fraction, previously obtained from HPLC-SEC. Green diagonal line represents the increasing acetonitrile gradient with respect to retention times. The 4 selected peptide peaks for further experimentation was named from 1 to 4, represented in blue.

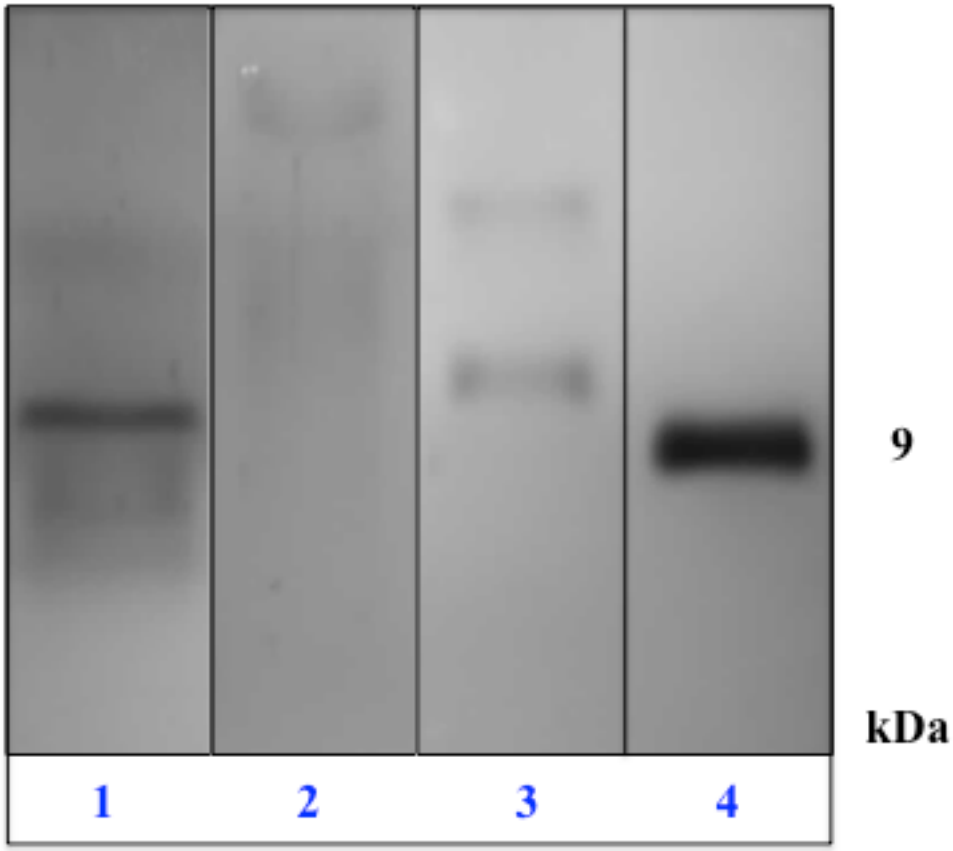

Figure 3.6 Tris-Tricine SDS-PAGE profiles of collected peptide fractions from HPLC-RP.

Tris-Tricine SDS-PAGE findings, again confirms the successful fractionation of venomic peptides. The cross-linking issue is still present and the 4th fraction of 9 kDa probably involves many other peptides. An important observation after these experimentations is the profiling of large peptides over 15 kDa, which were not previously present in the SDS-PAGE and MCE results of the crude venom and the peptide fraction. The rational explanation of this case may be low abundance, so that low concentration of large peptide masses in venom content. The sample loading concentrations and staining were done according to major peptide mass at

9 kDa for crude venom and SEC peptide fraction analysis. This may be masking the appearance of larger peptides at these figures.

3.5 Determination of Venomic Peptide Masses

To complete biochemical and peptidomic characterization of *L. abdullahbayrami* venom, determination of unique peptide masses is essential. To achieve this goal, SEC peptide fraction was analyzed by mass spectrometry. To prevent missing the detection of any masses, the peptide fraction introduced sequentially to mass spectrometry system by an in-line HPLC-RP system. Between the RTs 11 and 68 minutes over 90 minutes of total run, 45 probable peptide masses were identified (Table 3.2). These masses are at 1-7 kDa range. Just as in the case of HPLC-RP chromatogram, numbers of identified masses became denser between 25th and 55th minutes of the run. The complete mass chromatogram and a zoomed chromatogram to minute 22.56 can be seen in Figure C.2.

Table 3.2: Deconvoluted monoisotopic masses and their retention times of *L. abdullahbayrami* venom peptides detected by LC/MS TOF.

Retention Time (minutes)	Molecular Weight (Da)	Number of Masses
11.50	2961, 3000	2
12.04	3024	1
12.86	2948, 2988, 3183	3
15.39	3198	1
15.49	3234	1
20.90	3768	1
21.40	3772	1

(**Table 3.2** continued)

22.56	3996	1
23.37	4056, 4092, 4168	3
23.79	3996, 4000	2
24.19	4036	1
24.86	4056	1
25.66	6780	1
26.62	6805, 6820, 6840, 6855, 6895	5
29.14	4540, 6810	2
30.00	5376	1
33.32	3555, 3576, 3591, 3615, 3630	5
33.39	1067	1
34.65	1155	1
36.87	1287	1
36.95	1331, 1375	2
37.67	1419	1
38.20	1463	1
39.71	1507	1
43.05	1032	1
64.08	1294, 1338	2
64.15	1426	1
67.75	1082	1
	Total Mass Number	**45**

The molecular mass distribution of the identified peptides represented in Figure 3.7. As it can be seen, masses accumulate around three different groups as: around 1 kDa, between 3-5 kDa and in the range of 6-7 kDa's.

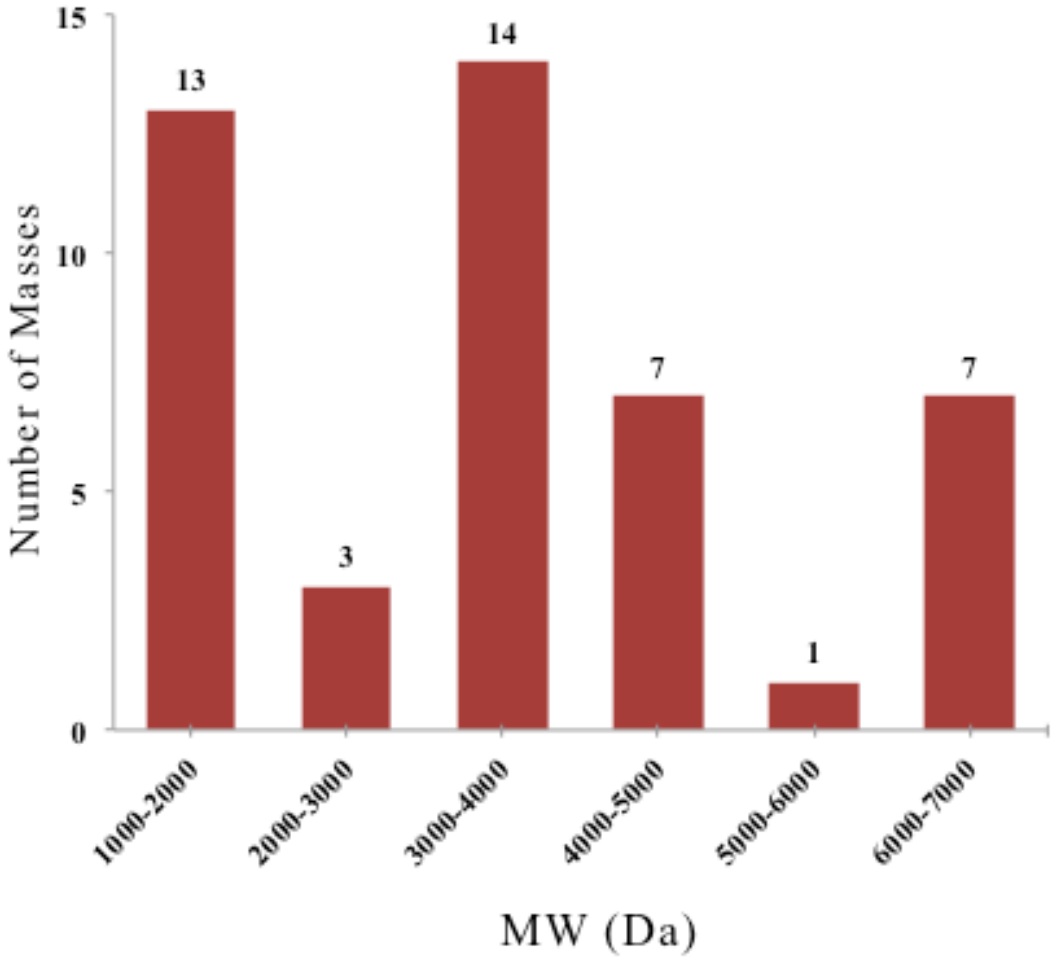

Figure 3.7 Distribution histogram of the venomic peptide molecular masses of *L. abdullahbayrami*.

According to this distribution trend and knowledge through the literature, a prediction could be done and those masses may be classified in three groups. The peptides at the range of 1-3 kDa's could be expected to be as NDBPs, which generally exhibit antimicrobial activities. The masses between 3-5 kDa's could be grouped in short-chain neurotoxins that follow the ICK motif. And lastly, peptides larger than 5 kDa may present the long-chain neurotoxins. Due to limitation of the LC/MS TOF analysis we may have omitted a portion of long-chain neurotoxins, which are heavier than 7 kDa. However, to our knowledge, the major portion (47%) of the peptide toxins of *L. abdullahbayrami* venom involves ICK motif short-chain neurotoxins that are generally functioning as K^+ channel blockers. The second major group (35%) consists of peptides without disulfide bridges or simply NDBPs. The identified part of the long-chain neurotoxins forms the 18% of the peptide toxins (Figure 3.8).

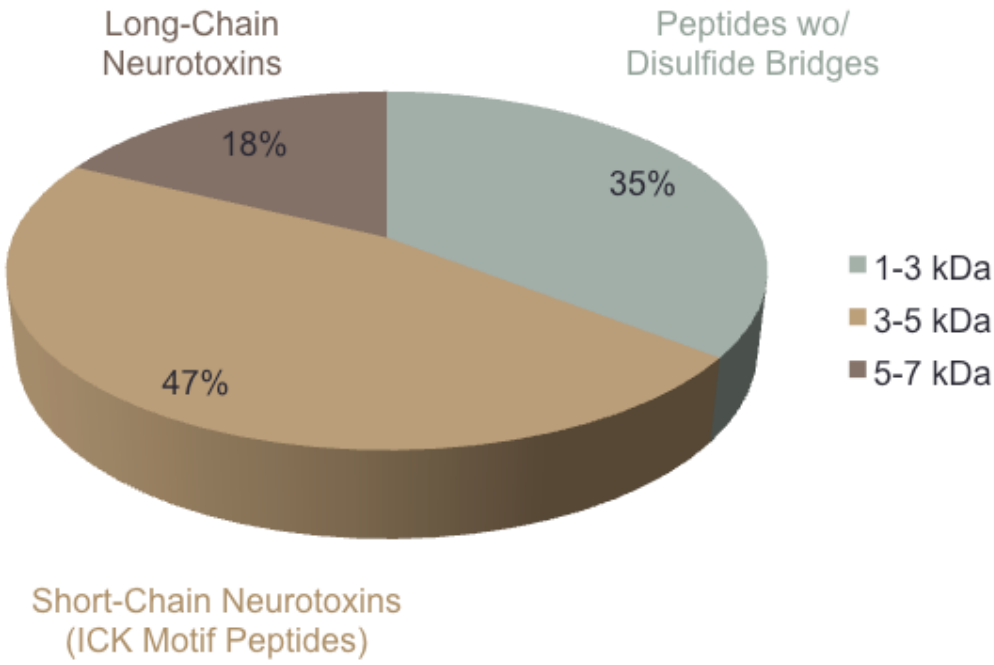

Figure 3.8 The estimated structural classification and dispersion of discovered peptide masses, represented in percentages in pie chart.

The most significant contribution of the mass spectroscopic analysis of venom peptide fraction may be the providing the first insight to presence of both long-chain and short-chain neurotoxins as well as NDBPs of the *L. abdullahbayrami* venom. As it has been mentioned numerous times, it was not possible to observe those peptides at theirs' expected MWs by electrophoretic methods. Moreover, obviously, mass spectroscopy comes with a remarkable advance over electrophoretic techniques.

3.6 Effect of *L. abdullahbayrami* Crude Venom on Cell Viability

Most of scorpion venom is composed of peptide neurotoxins with various bioactive properties, where the most abundant portions of those are ion channel inhibitor toxins. Ion channels, especially for K^+, are known to take important roles in cell proliferation. To identify *L. abdullahbayrami* venom's effect on cellular metabolism, MCF-7 breast adenocarcinoma cell line was used as model. The general expectation from venom's effect on a mammalian cell line is the inhibition of proliferation and decreased cell viability due to induction of apoptosis.

Maybe the most unexpected and therefore the most interesting part of this study is the viability assay. Treatment with a single dose of 200 µg/mL *L. abdullahbayrami* crude venom does not show any significant cytotoxicity on MCF-7 at 24 hours (Figure 3.9). On the other hand, a slight yet not significant increase in cell proliferation was observed. This observation persuades us to continue with time response experiments, where dose response was not achievable due to limitation of the venom amount. In 48 hours treatment with the same dose, as it had been suspected, *L. abdullahbayrami* crude venom showed a significant proliferative effect on the MCF-7 cell line (Figure 3.9).

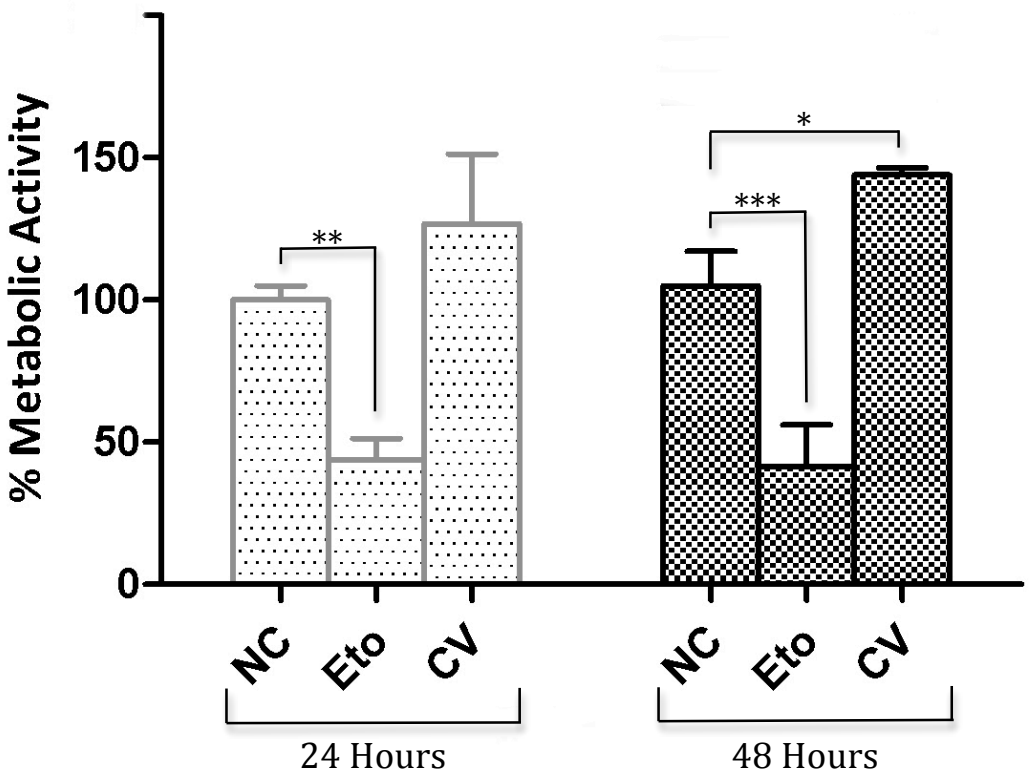

Figure 3.9 Viability assay of *L. abdullahbayrami* crude venom (200 µg/mL) on MCF-7 breast adenocarcinoma cell line.

The absence of any cytotoxic effect of a closely related species, *A. crassicauda*, was similarly shown on a number of cell lines (Caliskan et al. 2013). The proliferative effect of the crude venom may be due to low molecular weight components and ions of the crude venom, which were previously shown to have proliferative effect (Vassilevski et al. 2010). Another reason for this interesting finding can be originated from the free amino acid content of the crude venom. The cells may use these amino acids as nutrient, which are serum-depleted. Beside the number of discovered cytotoxic peptides are much more higher so far, a small

portion of venomic toxins inducing cell proliferation were screened. For instance a study identified mitogenic effect of venom fractions from very close relative, *L. quinquestriatus*, on Vero and BGM mammalian cell lines (Sherif et al. 2000). The indicated venom components in the precedents might be the reason behind the proliferative activity. Further experiments should be designed to address which venom fraction is taking role in the proliferative activity.

3.7 Antimicrobial Activity of *L. abdullahbayrami* Crude Venom

As it has been mentioned before, scorpion venom peptides were shown to contain antimicrobial peptides. To check antimicrobial activity of the crude venom, three gram negative, one gram positive as well as two pathogenic species from fungal origin were selected. The antibacterial activity was observed to be highest on gram negative species, where an indeed weak inhibition was also present for the gram positive strain. The highest inhibitory effect was on the *Escherichia coli* nearly 1.5 times higher than the positive control antibiotic, rifampin. The antimicrobial activity of crude venom on *Enterobacter aerogenes* is close to effect of tetracycline, a strong antibiotic. *Pseudomonas aeruginosa* was found to be most resistant gram negative strain to *L. abdullahbayrami* crude venom. *Listeria monocytogenes* showed strong resistance to crude venom. Crude venom screened to have mild antifungal potency against the two *Candida* species (Table 3.3).

The obtained antimicrobial profile of *L. abdullahbayrami* crude venom is comparable to its close relative *L. quinquestriatus* (Wesam & Naglaa 2014). The highlighted presence of NDBPs at 1-3 kDa range with mass spectroscopy analysis may be the essential players of the antimicrobial activity (Zeng et al. 2005). Further tests should be conducted on fractionated venom components, focusing on the low molecular weight peptides, to understand which peptides individually responsible for the observed activity.

Table 3.3 Antibacterial and antifungal activities of *L. abdullahbayrami* crude venom. Gram negative species, gram positive species and fungi represented in green, red and blue font colors respectively.

Microbial Strain	Zone of Inhibition (mm)		Antibiotic Disc
	Crude Venom	Antibiotic	
Escherichia coli	27	20	RA5
Enterobacter aerogenes	30	37	TE30
Pseudomonas aeruginosa	20	28	AMC30
Listeria monocytogenes	16	26	E15
Candida krusei	20	21	E15
Candida albicans	23	33	AMC30

CHAPTER 4

CONCLUSION

This study represents the first detailed biochemical characterization as well as bioactivity screening of *L. abdullahbayrami* venom. The electrophoretic profile and the venomic composition were determined by peptidomics approach, where the main focus was on the neurotoxins. Crude venom's effect on mammalian cell metabolism was investigated on MCF-7 cell line. The microbial growth inhibition by the *L. abdullahbayrami* venom was observed on a number of selected pathogenic microorganisms.

The protein content of the crude venom was found to be approximately 54% of the dry weight of the crude venom. The peptide and protein profiles of venom were determined by three different electrophoretic methods. This work not only presents the peptidomic/proteomic profile of *L. abdullahbayrami* venom, but also provides evaluation to those methods from a venomics approach due to its comparative nature. The intense peptide bands were observed in both techniques, as evidence for high abundance of peptide neurotoxins in the venom. *L. abdullahbayrami* venom was shown to be poor in terms of protein bands. MCE was seen to have a clear high-resolution advantage over other electrophoretic methods, while having essential limitations for the profiling of peptides smaller than 5 kDa.

The HPLC-SEC and HPLC-RP techniques were used for the fractionation of venomic components, where species-specific chromatograms from these methods

were also obtained. The major peptide peak of HPLC-SEC chromatogram again highlights the rich peptide content of the venom. The electrophoretic profiling of HPLC-RP fractions proves the presence of larger peptides resides at the range of 10 to 15 kDa in *L. abdullahbayrami* venom.

By mass spectrometric analysis of the venom peptide fraction, 45 unique peptide masses were discovered. These masses dispersed through 1 to 7 kDa, where expected NDBPs, short-chain neurotoxins and long-chain neurotoxins were detected. 60% of total peptide masses accumulated to 1-4 kDa range. Most abundant peptide class in terms of structure was estimated to be short-chain neurotoxins followed by the NDBPs and long-chain neurotoxins, respectively.

L. abdullahbayrami crude venom was shown to induce cell proliferation significantly on MCF-7 cells at 200 µg/mL dose and after 48 hours. Since the complete characterization of the venom was not completed, the venomic component that takes the essential role in this effect could not be identified. Yet ions, free amino acids, polyamines, biogenic amines or even a portion of peptides of the venom may be responsible for this phenomenon.

Crude venom of *L. abdullahbayrami* was screened to have high antibacterial activity on gram negative strains, while presenting poor effect on gram positive species. Moreover, a moderate antifungal activity on pathogenic *Candida* species, *C. albicans* and *C. krusei* were obtained. The mass spectrometry analysis indicated the presence of 1-3 kDa peptides, which are thought to be the non-disulfide bridged peptides having antimicrobial potentials. Those peptides should be the key players of strong antimicrobial potency of the *L. abdullahbayrami* crude venom.

This work may be considered as a preliminary study on the biochemical characterization and bioactivity screening of *L. abdullahbayrami* venom. The

original source of proliferative and antimicrobial effects may be addressed to venom components or individual molecules. The identification, further characterization and molecular engineering of these components may lead to the development of new therapeutic or diagnostic tools, such as peptide antibiotics. The ion channel modulator peptide toxin sequences from *L. abdullahbayrami* venom can also be deciphered, which may in turn lead to discovery of novel Na^{+}, K^{+}, Ca^{2+} or Cl^{-} channel inhibitors. These can be used as core molecular scaffolds for the development of new drugs

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APPENDIX A

MEDIUM SPECIFICATIONS

Table A.1 DMEM 4.5 g/L D-glucose with L-glutamine

Compound	Concentration (mg/L)
Calcium Chloride Anhydrous	200.000
Dextrose	4,50E+06
Ferric Nitrate Nonahydrate	0.100
Magnesium Sulfate Anhydrous	97.670
Potassium Chloride	400.000
Sodium Bicarbonate	3,70E+06
Sodium Chloride	6,40E+06
L-Arginine Monohydrochloride	84.000
L-Glutamine	584.000
Glycine	30.000
L-Histidine Monohydrochloride Monohydrate	42.000
L-Isoleucine	104.800

(**Table A.1** continued)

L-Leucine	104.800
L-Lysine Monohydrochloride	146.200
L-Methionine	30.000
L-Phenylalanine	66.000
L-Serine	42.000
L-Threonine	95.200
L-Tryptophan	16.000
L-Valine	93.600
D-Calcium Pantothenate (Vitamin B5)	4.000
Choline Chloride	4.000
Folic Acid	4.000
I-Inositol	7.000
Niacinamide (Nicotinamide)	4.000
Pyridoxine Monohydrochloride	4.000
Riboflavin (Vitamin B2)	0.400
Thiamine Monohydrochloride (Vitamin B1)	4.000
Phenol Red	15.000
Pyruvic Acid Sodium Salt	110.000
L-Tyrosine Disodium Salt, Dihydrate	103.790
L-Cystine Dihydrochloride	62.580
Sodium Phosphate Monobasic, Anhydrous	108.690

Table A.2 Mueller Hinton broth medium

Compound	Concentration (g/L)
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5

Table A.3 Mueller Hinton agar medium

Compound	Concentration (g/L)
Beef infusion solids	4.0
Starch	1.5
Casein hydrolysate	17.5
Agar	15.0

Table A.4 Sabouraud dextrose-agar medium

Compound	Concentration
	(g/L)
Mycological Peptone	10.00
Dextrose	40.00
Agar	15.0

Table A.5 1X PBS Solution

Compound	Concentration
	(mg/L)
Potassium Phosphate monobasic (KH2PO4)	210.0
Sodium Chloride (NaCl)	9000.0
Sodium Phosphate dibasic (Na2HPO4-7H2O)	726.0

APPENDIX B

SOLUTIONS

1X Tris-Glycine Running Buffer

12.11 g Tris

57.6 g Glycine

40 mL 10% SDS Solution

Dissolved in dH_2O and volume was adjusted to 4 L. Stored at room temperature.

Separating Gel Buffer

181.65 g Tris

40 mL 10% SDS Solution

800 mL dH_2O

pH was adjusted with HCl to 8.8 and volume was completed to 1 L. Stored at room temperature.

Stacking Gel Buffer

181.65 g Tris

40 mL 10% SDS Solution

600 mL dH_2O

pH was adjusted with HCl to 6.8 and volume was completed to 1 L. Stored at room temperature.

Table B.1 Tris-Glycine SDS-PAGE gel solution preparations from stock solutions

		4% Stacking Gel	**10% Separating Gel**
AB-3 Solution	mL	0.625	2.5
Stacking Gel Buffer	mL	0.42	-
Separating Gel Buffer	mL	-	2.5
Adjust with dH_2O to Final Volume	mL	5	10
Polymerize by Adding			
10% APS Solution	µL	30	50
TEMED	µL	6	10

1X Cathode Buffer

12.11 g Tris
17.92 g Tricine
10 mL 10% SDS Solution
Dissolved in dH_2O and volume was adjusted to 1 L. Stored at room temperature.

1X Anode Buffer

12.11 g Tris
800 mL dH_2O
pH was adjusted with HCl to 8.9 and volume was completed to 1 L with dH_2O. Stored at room temperature.

AB-3 Solution

120 mL 40% acrylamide solution

1.8 g bisacrylamide

Mixed and stored at room temperature.

AB-6 Solution

116.25 mL 40% acrylamide solution

3.5 g bisacrylamide

Mixed and stored at room temperature.

3X Gel Buffer

363.2 g Tris
30 mL 10% SDS Solution
600 mL of dH_2O
pH was adjusted with HCl to 8.45 and volume was completed to 1 L with dH_2O. Stored at room temperature.

10% SDS Solution

50 g SDS
Dissolved in dH_2O and volume was adjusted to 500 mL. Stored at room temperature.

10% APS Solution

1 g APS
Dissolved in dH_2O and volume was adjusted to 10 mL. Stored at -20^{o}C.

Table B.2 Tris-Tricine SDS-PAGE gel solution preparations from stock solutions

		4% Stacking Gel	**10% Spacer Gel**	**16% Separating Gel**
AB-3 Solution	mL	0.4	1.2	-

(**Table B.2** continued)

AB-6 Solution	mL	-	-	3.8
3X Gel Buffer	mL	1	1.67	3.3
Urea	g	-	-	3.6
Adjust with dH_2O to Final Volume	mL	4	5	10
Polymerize by Adding				
10% APS Solution	µL	30	25	35
TEMED	µL	6	5	7

Gel Fixing Solution

150 mL methanol

36 mL acetic acid

150 µL formaldehyde

114 mL dH_2O

Stored at room temperature.

50% Ethanol Solution

500 mL ethanol

500 mL dH_2O

Stored at room temperature.

Pre-Treatment Solution

50 mg sodium thiosulfate

Dissolved in dH_2O and final volume adjusted with dH_2O to 200 mL. Prepared freshly before use.

Silver Nitrate Solution

500 mg silver nitrate

188 µL formaldehyde

Dissolved in dH_2O and final volume was completed to 250 mL with dH_2O. Prepared freshly before use.

Developing Solution

5.625 g potassium carbonate
5 mL pretreatment solution
188 µL formaldehyde

Dissolved in dH_2O and final volume was completed to 250 mL with dH_2O. Prepared freshly before use.

Stop Solution

200 mL Methanol
30 mL Acetic Acid
170 mL dH_2O
Stored at room temperature.

SEC Running Buffer

100 mL acetonitrile

1 mL TFA

899 mL dH_2O

Prepared freshly before use. Degassed by sonication and helium sparging.

Solution A for Reversed Phase Chromatography

1 mL TFA

999 mL dH_2O

Prepared freshly before use. Degassed by sonication and helium sparging.

Solution B for Reversed Phase Chromatography

1 mL TFA

999 mL acetonitrile

Prepared freshly before use. Degassed by sonication and helium sparging.

Solution A for LC/MS TOF

1 mL formic acid

999 mL dH_2O

Prepared freshly before use. Degassed by sonication and helium sparging.

Solution B for LC/MS TOF

1 mL formic acid

999 mL acetonitrile

Prepared freshly before use. Degassed by sonication and helium sparging.

APPENDIX C

ADDITIONAL FIGURES

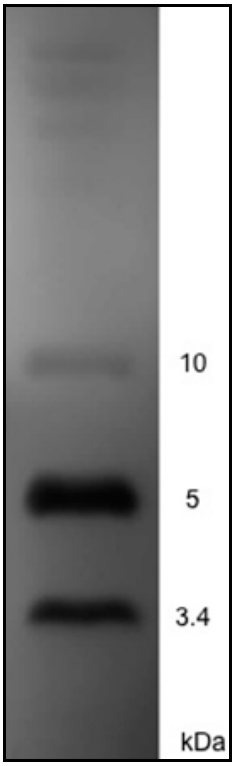

Figure C.1 Separated protein ladder in 16% Tris-Tricine SDS-PAGE.

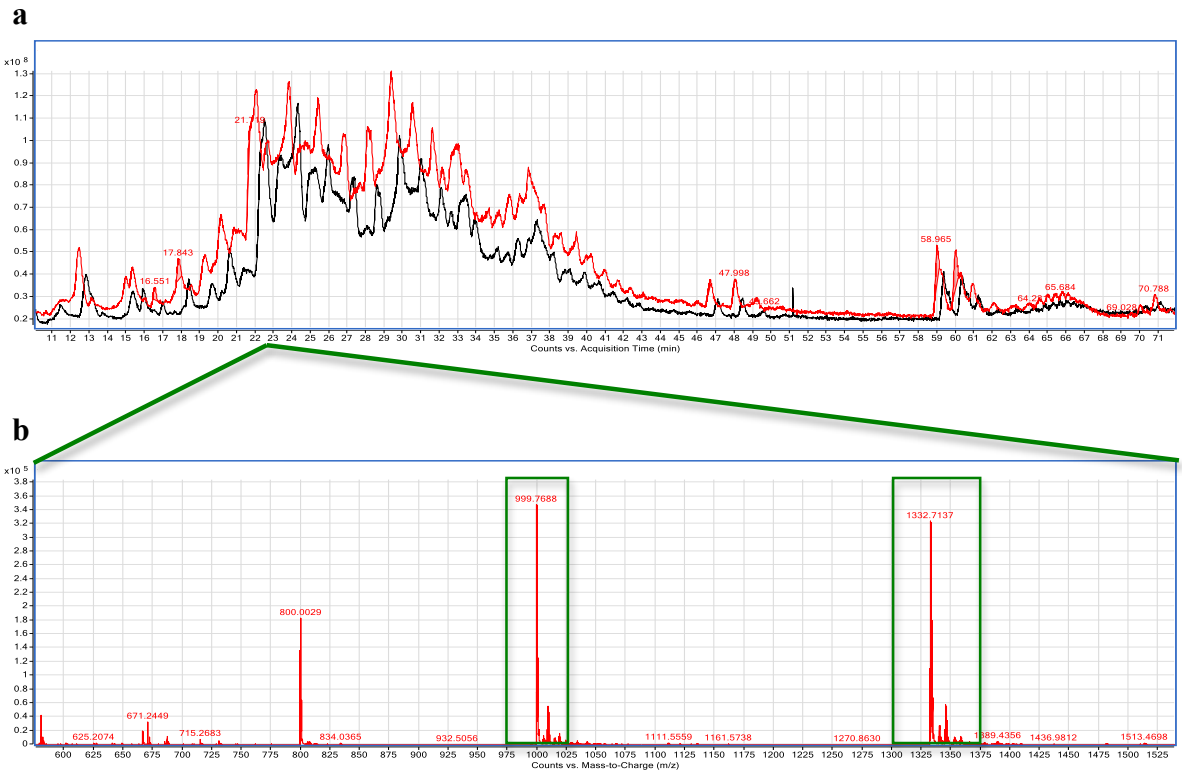

Figure C.2 (a) Mass chromatogram of *Leiurus abdullahbayrami* venom peptide fraction from second 11 to 70. (b) Zoomed chromatogram at 22.56th second, observed peaks are marked in green. m/z ratios of the same mass (3996 Da) in different charges can be seen at the top.