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**Original Article**

# A Fast Method for Detection of Fake Venoms and Investigation of Interspecies Variation Using Venoms of *Androctonus*, *Buthus*, and *Leiurus* (Scorpiones: Buthidae) Species

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**Abstract**

**Background:** Within the scorpion family Buthidae, some of the most dangerous venomous genera are *Androctonus* (*A*), *Buthus* (*B*), and *Leiurus* (*L*). This venom is valuable raw material for numerous therapeutic formulations because of its pharmacological potential; however, because of its high prices in the global market, fake "venom mixes" are being made to market illegally, and it is important that these unknown mixes be evaluated. A fast and accurate response to the request for this identification is necessary.

**Method:** This study was conducted in Turkey in 2022. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is a linear positive-ionization mode was used for identification of scorpion species. The mass spectra of the three scorpion venoms were examined in detail. The peptide and protein profiles in the venoms of congenerous three scorpion venoms and the proportional differences in these venoms were investigated. For interspecific variation, a principal component analysis of all venoms was conducted, and variance values and distance-proximity indices were determined.

**Results:** The top three peptide masses in the highest relative abundance for *A. australis*, *B. mardochei*, and *L. quinquestriatus quinquestriatus*, respectively, were 6901, 7431, and 7447; 4238, 5283, and 4055; and 3828, 7868, and 6799 Da. While the variance rate between *A. australis* and the other two venoms was 40%, this rate was 38% between *B. mordochei* and *L. quinquestriatus quinquestriatus* venoms.

**Conclusion:** A very simple protocol of species identification using scorpion venom samples was created using recent advances in MALDI-TOF MS.

**Keywords:** *Androctonus australis;* Buthidae; *Buthus mardochei; Leiurus quinquestriatus quinquestriatus*; Scorpiones

## Introduction

Scorpions are predatory arachnids which live in all parts of the world except the Polar Regions and some islands (1). Scorpion stings are common in many countries each year and although most stings cause only localized pain without life-threatening poisoning, about one-third cause systemic poisoning, which can be fatal, with children being the most susceptible.

The severe effect of scorpion stings is associated with the presence of neurotoxins that predominantly affect the sympathetic nervous system in mammals and cause a sudden release of neurotransmitters from the autonomic nervous system. Many vital functions can be directly affected, including the cardiovascular, respiratory, and neuromuscular systems (2). Most cases of poisoning are caused by stings from some species of scorpions who belong to the Buthidae family and are known for their deadly venom (3).

Buthidae is the largest and most important family of interest to the medical and veterinary professions, and represents near one-half (48%) of the known scorpion species, including those who are the most dangerously venomous.

The venom of these family members contains a heterogeneous cocktail of compounds that comprise inorganic substances, enzymes, mucopolysaccharides, allergenic compounds, and peptides and are highly toxic to the ionic channels of excitable cells (4). These venom fractions contain specific neurotoxins, such as acetylcholinesterase, hyaluronidase, serotonin, and phospholipase. Typically, these neurotoxins cause the ion channels to open at the neuromuscular junction, and repetitive nerve depolarization leads to the activation of the sympathetic and parasympathetic nervous systems, resulting in the release of catecholamines and acetylcholine (5).

The more dangerous genera within the Buthidae family are *Androctonus* (defined as yellow fat-tailed scorpion), *Buthus*, and *Leiurus* in northern Africa and western Asia. The venom from *A. australis* (Linnaeus, 1758) is known for its high toxicity to humans (6).

With the knowledge of the components of scorpion venom, the venom can have pharmacological potential as the valuable raw material for numerous therapeutic formulations and holds an important place in the global market. These scorpion toxins are classified into the following four channels according to their pharmacological targets: sodium (I), potassium (II), chloride (III), and calcium (IV), and several or their derivatives are being developed into drugs by major pharmaceutical companies. For example, chlorotoxin, which targets chloride channels, is being considered for the treatment of glioma cancer (7).

Unfortunately, because of their high costs, fake "venom mixes" are being illegally marketed; therefore, it is important to determine whether any unknown compounds being marketed are actually scorpion venom.

In the present study, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis method was developed for rapid identification and discrimination of scorpion species with each other and their venoms with other venoms. A need was recognized to develop a fast and accurate response to requests in our center, Turkish Public Health Institute, for the protection of the public for the identification of mixes of unknown content that are represented as venom. Within this context, MALDI-TOF MS can be a quick and simple tool by which to identify and distinguish the characteristics among scorpion venoms. In our previous studies, MALDI-TOF MS was used to identify *Androctonus crassicauda* and *Leiurus abdullahbayrami* scorpions, who belong to Buthidae and live throughout Turkiye (8,9).

In the present study, peptide and protein distributions, as well as spectra of each of the three species, were evaluated using MALDI-TOF MS to identify the venoms of *A. australis*, *B. mardochei* and *L. quinquestriatus quinquestriatus (L. q. quinquestriatus*), also members of Buthidae family, and the venoms belonging from these three species were differentiated from each other.

## Materials and Methods

This study was conducted in Turkey in 2022. Three scorpion venom standards used in the present study were purchased from Latoxan (Portes-lès-Valence, France) and were 99.9% pure. Venom standards with codes of L2130 (batch # 511.191) comprised 743 *A. australis* donor scorpions of Egyptian origin, L2107 (batch # 125.031) comprised 15,665 *B. mardochei* donor scorpions from Morocco, and L2111(batch # 727.051) comprised

1,571 *L. q. quinquestriatus* donor scorpions of Egyptian origin.

*Escherichia coli* bacterial test solution (BTS) which was used for the internal quality control process, and alpha-cyano-4-hydroxy cinnamic acid (α-CHCA), which was a MALDI matrix, were purchased from Bruker (Bremen, Germany). Acetonitrile (ACN, high performance liquid chromatography grade; Sigma-Aldrich, St. Louis, MO, USA), trifluoroacetic acid (TFA; Sigma-Aldrich), and 0.1 µm filtered ultrapure water (UPW) free of DNAase and RNAase were used to prepare the matrix solution.

### *Preparation of scorpion venom-matrix solutions*

The total protein amount in the three scorpion venoms was measured using Nano Ready Touch (Life Real Biotechnology Co., Ltd., Hangzhou, China) at 280 nm and prepared using UPW to equal 2 mg/mL. All venom was then centrifuged for 15 min at 15000 rpm and +4 ºC. The supernatants were transferred into individual polypropylene Eppendorf tubes to which 250 µL matrix solution was added (18 mg/mL α-CHCA, 50% ACN, and 2.5% TFA mixture; 1:1; v/v) to each. Six ([n = 3] x 2) scorpion venom matrix samples (SVMx) prepared in parallel were vortexed at 3000 rpm for 2 min.

### *Acquisition of MALDI TOF MS*

The internal quality control process for MALDI-TOF MS measuring seven peaks was completed (m/z, RS32 $[M+H]^+$: 5096.5112 Da; RS34 $[M+H]^+$: 5381.2220 Da; RS33meth $[M+H]^+$: 6256.0007 Da; RL29 $[M+H]^+$: 7274.7831 Da; RS19 $[M+H]^+$: 10299.5137 Da; RNAase A $[M+H]^+$: 13683.2401 Da, and Myoglobin $[M+H]^+$: 16952.4305 Da) with a standard deviation of 55.275 and a maximum peak error of 75.50 ppm.

A 1-µL sample of SVMx from each scorpion venom was spotted in parallel on a custom steel 96 micro-scout plate (MALDI 96 MSP; Bruker Daltonics, Bremen, Germany) into three separate wells, and allowed to dry completely at room temperature. The MALDI 96 MSP loaded with the samples was placed in the Microflex-LT MALDI-TOF MS (Bruker Daltonics, Bremen Germany) and operated in linear positive-ion mode within the mass range of 1–50 kDa; a 60 Hz nitrogen laser at 337 nm was used as the ion source. To obtain the spectra, 400 laser pulses consisting of 40 packets were applied. Six spectra were collected for each scorpion venom. Each spot sample was examined in triplicate and the reads with the highest relative abundance were included in the analyses.

### *Flex-analysis software: visual inspection*

A visual inspection of all spectra from the three scorpion venoms was conducted using flexAnalysis software v. 3.4 (Bruker Daltonics; after smoothing, baseline subtraction) to detect species-specific ions. Sensitivity and specificity values were calculated for each potential subspecies-specific mass peak.

Because each mass signal represented a single molecular dimension within the mass spectra, they were considered as multivariate data, and multivariate statistical methods were used to distinguish the samples (10).

### *Principal component analysis using MATLAB software*

The aim of principal component analysis (PCA) is to reduce the dimensionality of the dataset having a large number of interrelated variables while retaining variation. In mass spectra, the intensities of individual m/z ratios represent the variables. PCA calculates new coordinate system data that can be expressed as a linear combination of the original variables (mass-to-range ratios m/z). This enables major trends in the data to be identified.

MATLAB software (Bruker Daltonics) has provided an alternative for data management. MALDI-TOF MS spectra is used to recognize potential biomarkers. MATLAB software allows the creation of models for automatic classification based on differences in the spectra and supports the visual inspection of broader spectrum cohorts, facilitating the detection of peaks that distinguish among the spectral groups evaluated.

By creating a dendrogram profile with PCA, a separate cluster of the three scorpion venoms was created. The projections of peptide and protein

(P&P) peaks in the scorpion venoms were macroscopically evaluated by creating a virtual gel image (VGI). P&P peaks in the spectra can appear as lines in VGI according to the relative abundance (RA) values. As the RA% increases, the vertical lines become thicker and their color changes from light blue to red (light blue: low RA; red: highest RA). The spectra were massed using the PCA method supported by external MATLAB software using the MALDI Biotyper method. Optimized preliminary procedures were applied for each spectrum to accelerate the analysis and reduce the data mass dimension. The raw mass spectra were preprocessed using Biotyper software v. 3.1 before further analysis based on the Biotyper preprocessing standard method (smoothing method: Savitski-Golay; baseline subtraction method: multi polygon; normalization method) (11).

## Results

### *Species-level identification of MALDI-TOF MS scorpion venoms*

MALDI-TOF MS analyses were conducted within the mass range of 1–50 kDa of three scorpion venoms *A. australis*, *B. mardochei and L.q. quinquestriatus*, each of which is a member of Buthidae, and their mass spectra and peptide and protein distributions were evaluated. The mass spectrum, unique to each venom, consisted of laser-ionized P&P molecules. For the analyses of P&P distributions within the three scorpion venoms, the highest RA within the spectrum was accepted as 100%, and the RA% of the other molecules was calculated separately.

The *A. australis* spectrum contained approximately 112 molecular masses ranging from 1202 to 33523 Da. The majority of these (80 pieces) were peptide molecules. Fig. 1A presents the spectrum of the P&P profile of *A. australis* scorpion venom mix in detail. In general, it was observed that peptide molecules were dominant within the range of 3000–8500 Da in the spectrum, but it was also observed that some protein molecules were >10 kDa. In addition, VGI were created, and the P&P distribution was examined (Fig. 1B). VGI provided information about which peaks were dominant in which regions within the range of 2–20 kDa. Peptides with a high relative abundance within the mass spectrum (6901 Da, 100% RA; 7431 Da, 96% RA; 7447 Da, 93% RA; 7344 Da, 91% RA; and 8469 Da, 82% RA) were also seen in VGI. Among the peptides between 3000 and 4300 Da (K channel blockers), there was a relative abundance of peptides of 3445 Da (60% RA) and 3670 Da (50%) (Fig. 1).

*B. mardochei* scorpion venom contained approximately 105 molecules ranging in mass from 1284 to 16518 Da and that most of these molecules (103) were peptides.

Approximately 60% of all peptide molecules in *B. mardochei* scorpion venom were concentrated within the range of 1000–5000 Da (Fig. 2A). Peptides with RA values >40% (3300 Da, 45%; 4037 Da, 72%; 4212 Da, 69 % and other two peptides) were within this region, especially those between 3200 and 4300 Da. Five peptide molecules with >40% RA within the range of 5000–7300 Da were also present (Fig. 2B).

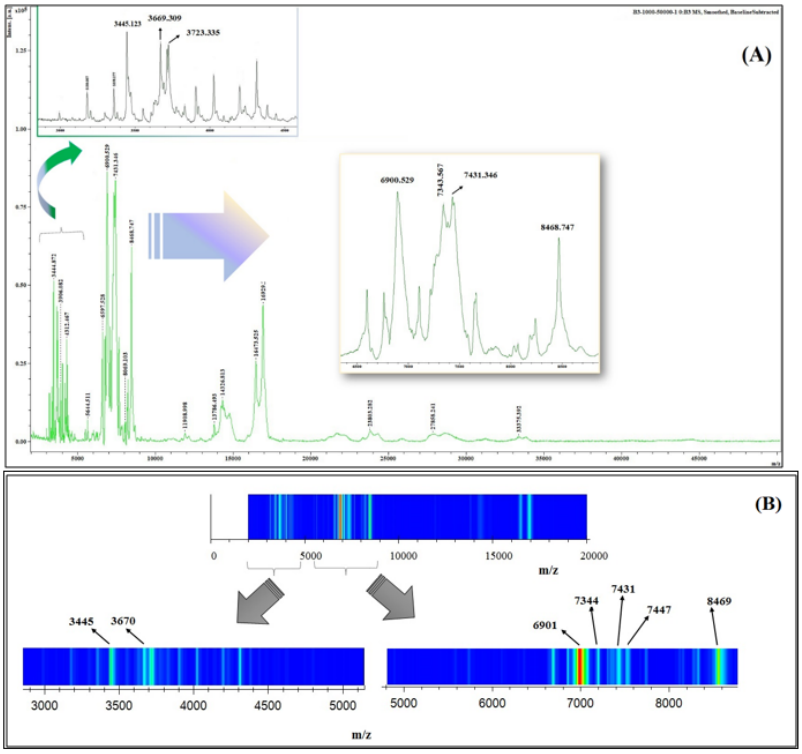


**Fig. 1:** A) Representative matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and B) virtual gel image of *Androctonus australis*

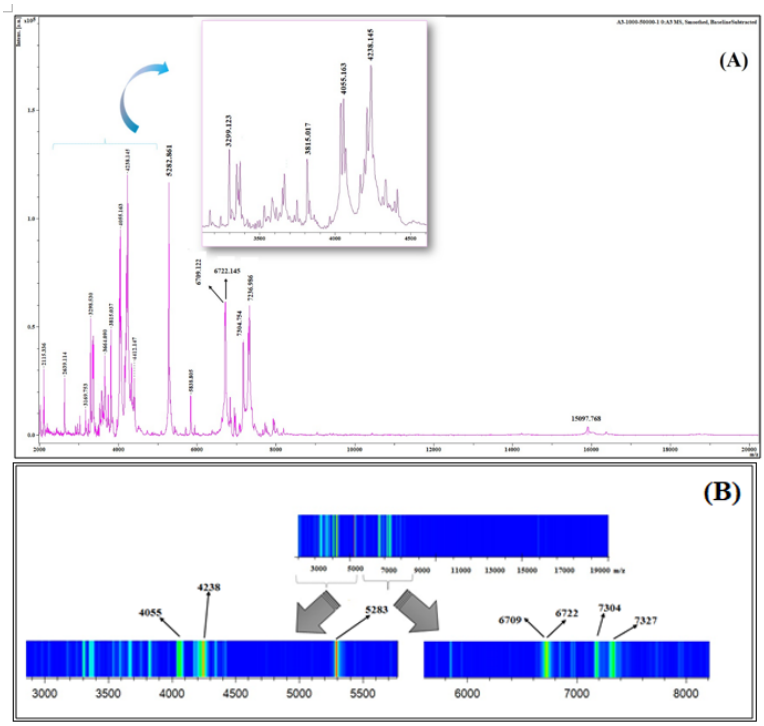


**Fig. 2:** A) Representative matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and B) virtual gel image of *Buthus. mardochei*

*L.q. quinquestriatus* scorpion venom contained approximately 100 molecular masses ranging from 1215 to 28310 Da and 81% of these molecules were peptides. As seen in the mass spectrum in Fig. 3A, high-RA peptide molecules were abundant in both the 3000–5000 and 5000–8500 Da ranges. High abundance peptides (3402 Da, 69%; 3283 Da, 100%; 3948 Da, 58%; 4003 Da, 70%; 6621 Da, 51%; 6623 Da, 66%; 6799 Da, 76%; 6843 Da, 57%; 6557 Da, 57%; 7203Da, 56%; 7299 Da, 65%; 7328 Da, 55%; and 7868Da, 94%) were observed as thick green and red bands in VGI (Fig. 3B).

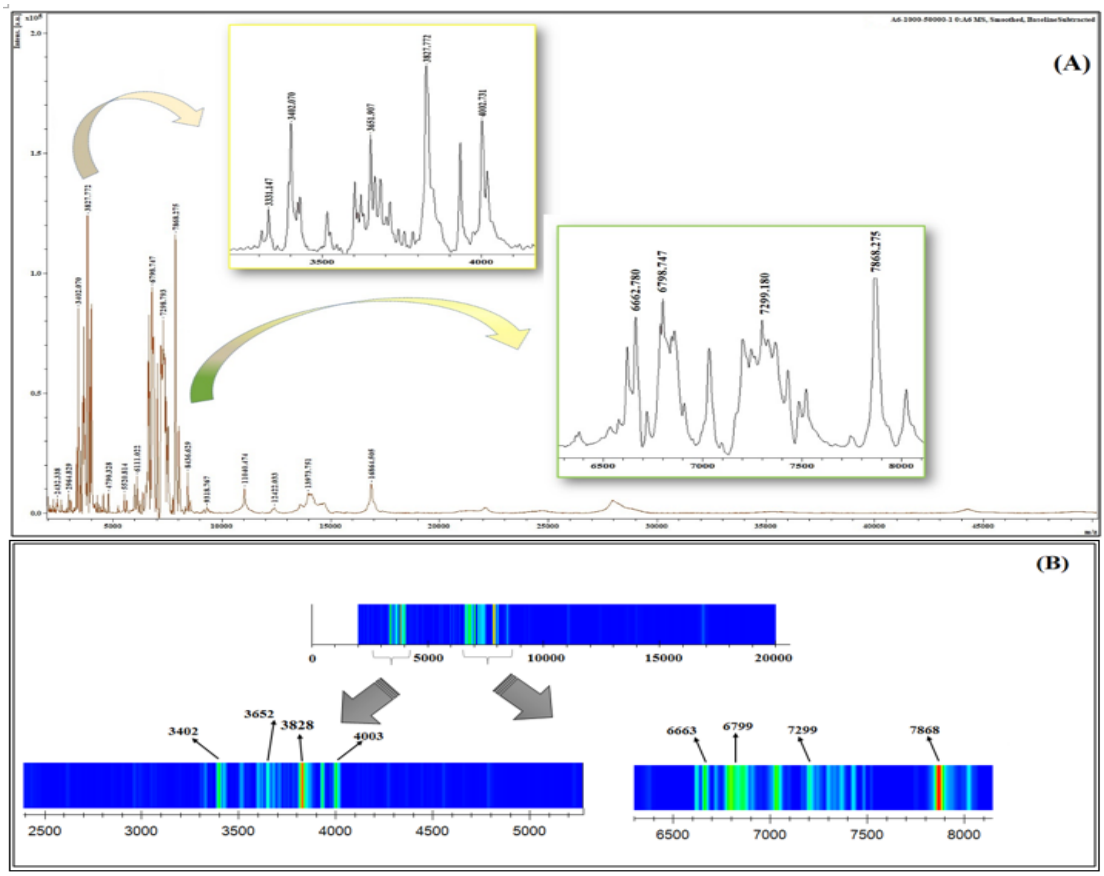


**Fig. 3:** A) Representative matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and B) virtual gel image of *Leiurus quinquestriatus quinquestriatus*

### *Interspecies variance and comparison of peptide and protein distributions of three scorpion venoms*

The venom from three scorpion species (*A. australis*, *B. mardochei*, and *L.q. quinquestriatus*) of the Buthidae family was analyzed using MALDI-TOF MS. As mentioned, six spectra were created for each scorpion venom. PCA analyses (dendrogram, 2D-scatter plotting, 2D-mass loading profile, variance explained profile) were conducted using MATLAB software on the unique spectra of the three scorpion venoms and were presented in Fig. 4. In the dendrogram profile, six distinct spectra belonging to the same scorpion venom were placed in the same group, forming three clusters for three separate scorpion venoms (Fig. 4A). Similarly, in the 2D scattering profile, where each dot represents the spectrum, members of the same scorpion venom were found in each of the three separate clusters (Fig. 4B). The mass scattering profile is shown in Fig. 4C. At the center of this 2D mass loading profile (yellow rectangular area),

which is a projection of the cluster distribution in Fig. 4B, each dot corresponds to an ionized peptide or protein molecule and is of similar value within the three scorpion venoms. The greater the distance of a peak from its origin, the greater its contribution to the variance of the dataset in the mass loading profile (Bruker, MALDI Biotyper 3.0 User Manual) (Fig. 4D). For example, the molecular mass of 6902 Da represented the most abundant peptide in *A. australis* scorpion venom, was far from the center, and was not present in the other two scorpion venoms. Similarly, the molecular mass of 4238 Da in the venoms of *B. mardochei* and 3828 Da in *L.q quinquestriatus* represented the most abundant peptide in those venoms and were also located very far from the center.

In addition, Fig. 4D provides the P&P distributions (α, β, and γ) and RA% and pie distributions (i, ii, and iii) of the three scorpion venoms. Accordingly, peptide distributions in *L.q. quinquestriatus* venom between 3 and 5 and 5 and 9 kDa were ≥35% (i in Fig. 4D) and had high RA% (α in Fig. 4D). In *B. mardochei* venom, peptide molecules between 3 and 5 kDa had the highest rate (47%), followed by peptides within the range of 5–9 kDa (39%) (ii in Fig. 4D). Compared to the other two venoms, the distribution ratios of the peptides in the *A. Australis* venom were similar to each other based on the mass ranges in the pie profile (24% for 1–3 kDa; 22% for 3–5 kDa; and 26% for 5–9 kDa) in Fig. 4D (iii). In addition, peptides with a high RA% were mostly within the range of 5–9 kDa (γ in Fig. 4D).

An analysis of variance was conducted for the three scorpion venoms. While the variance rate between *A. australis* and the other two venoms was 40%, this rate was 38% between *B. mordochei* and *L.q. quinquestriatus* venoms. As seen in the variance profile in Fig. 4E, the very low variance values from PC3 to PC9 represented the high in-group similarity of each scorpion venom (Figs. 4A, 4E).

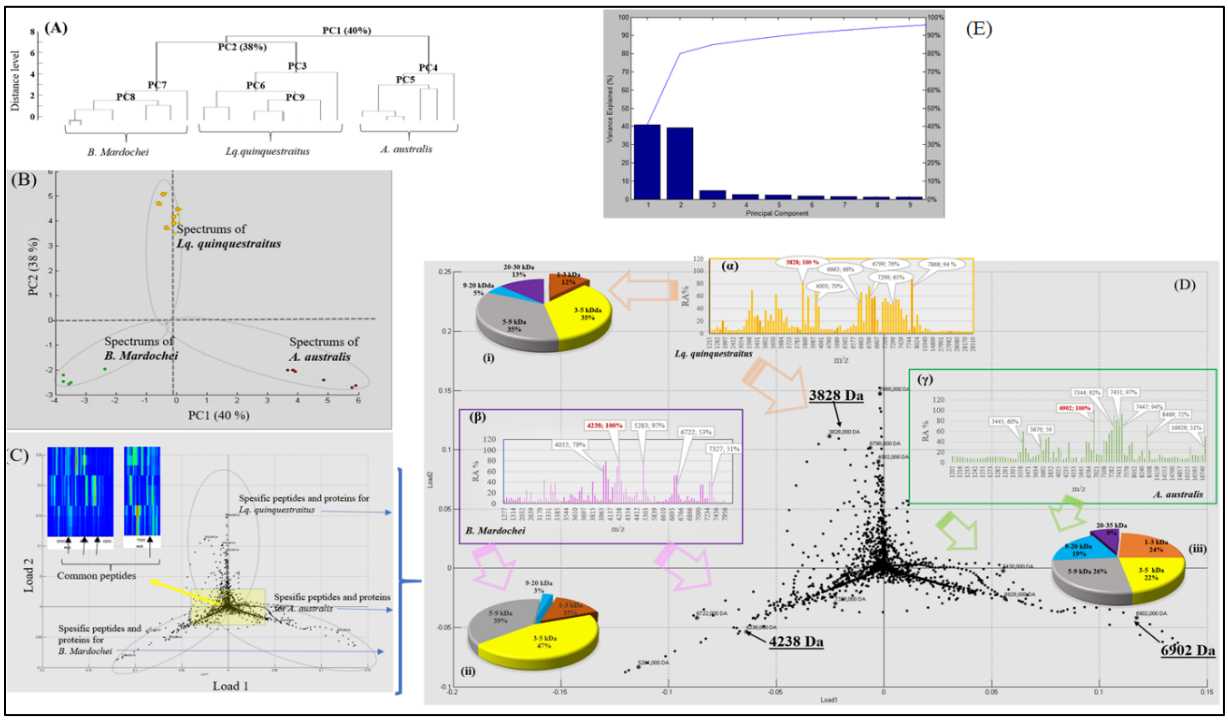


**Fig. 4**: Principal component analysis of three scorpion venoms (*Androctonus australis, Buthus mardochei, and Leiurus quinquestriatus quinquestriatus*). A) dendrogram; B) 2D-scatter plot profile; C) 2D-spectra loading; D) detailed spectra loading, both percent relative abundances of peptides and proteins(P&P) in three venoms (α, β, γ) and pie distributions of P&P of three venoms; and E) variance

## Discussion

The toxicity of scorpion venoms is the result of the peptides and proteins they contain (12). Many studies have been conducted for the identification of scorpion venoms(13–22). Although most of these were investigated the pharmacological properties of Buthidae scorpion venoms (7,14,15,17,19,23–25), some recent studies focused on the identification and characterization of Buthidae scorpion venoms using state-of-the-art devices (4,26–30).

In the present study, the mass profiles of three scorpion venoms (*A. australis*, *B. mardochei,* and *L.q. quinquestriatus*), all members of the Buthidae family, were characterized in detail by creating scanned mass spectra within the range of 1–50 kDa using MALDI-TOF MS. Because of the high relative abundance values of peptides in all three scorpion venoms with masses <10 kDa, differentiation among the species was made using these peptide molecules.

Manual examination of the peaks across the spectra using flexAnalysis software v 3.4 (Bruker Daltonics) and macroscopic examination using VGI and MATLAB software revealed the presence of at least five distinctive peaks belonging to the three scorpion venoms. Moreover, MATLAB software was used to confirm the differentiation by finding the molecular peaks that were present and different in each scorpion's venom on the 2D-scatter-loading profile.

The results obtained using the MALDI-TOF MS method, in which the molecular ions were formed by soft ionization of intact proteins, were compared with those of the research using the mass spectrometric method.

For example, Daoudi et al. (4) conducted an LC-ESI MS/MS analysis of *B. occitanus* venom and some of the peptide molecules (4333, 4366, 6873, 6973, 7079, 7115, 7177, 7250, 7308, and 7390 Da; m/z) detected were the same as those detected in *B. mardochei* venom as molecular peptides (6873, 6973, 7079, 7115, 7177, 7250, 7308, and 7390 Da; m/z) found in the present study. In addition, some of the peptide molecules (2025, 3636, 3666, 3732, 3768, 6947, and 7308 Da; m/z) determined by Martin-Eauclaire et al. (14) were found to match with the peptides detected in the present study. Furthermore, it is noteworthy that the peptide molecule with a value of 7308 Da found in 50% abundance in the present study was also detected in both of the above-mentioned studies (4,22). Finally, a molecular peptide of 3764 Da with the highest signal intensity detected by Schaffrath et al (31) in *Buthus occitanus* analysis using MALDI-TOF MS was also detected in the present study.

Studies on both *A.ausralis* and *L.q. quinquestriatus* venom have focused mainly on its channel blocker ($K^+$ and $Na^+$) properties and its therapeutic use (14,23,24,31). Al-Asmari et al (31) have conducted an elemental analysis of *L.q. quinquestriatus* venom using ICP-MS. Sarhan et al (30), on the other hand, have conducted a study on the genetic diversity of *L.q. quinquestriatus* populations.

## Conclusion

The primary aim of the present study was to identify scorpion venoms as easily, quickly, and repetitively as possible. For the creation of an in-house database of scorpion venoms with definitions, MALDI-TOF MS was used in linear positive-ionization mode. The results indicated that this method would be useful in identifying an unknown substance as scorpion venom and providing preliminary information about its origin. Interacting with MALDI-TOF mass spectra can serve as a suitable basis for creating a taxonomic scorpion toxin database. Of the numerous peptide mass signals detected using this method, very few will be sufficient for species recognition. MALDI-TOF MS can help discriminate among species when the method is combined with specific biomarkers. This manuscript describes a very simple protocol for using scorpion venom samples for species identification, and considers recent advances in MALDI-TOF MS.

## Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the author.

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## Conflict of Interest

The author has declared no conflict of interest related to the present study or its results.

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