Analytical methods for honeybee venom characterization

Iouraouine El Mehdi1,2,3, Soraia I. Falcão3, Said Boujraf4,5, Harandou Mustapha1, Maria G. Campos4,5, Miguel Vilas-Boas3
1Prof. Said, Clinical Neurosciences Laboratory, Faculty of Medicine and Pharmacy, 2Department of Biophysics and Clinical MRI Methods, Faculty of Medicine and Pharmacy, University Sidi Mohamed ben Abdellah, Fez, Morocco, 3Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Bragança, 4Observatory of Drug-Herb Interactions, Faculty of Pharmacy, University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba, Coimbra, 5Coimbra Chemistry Centre (CQC, FCT Unit 313) (FCTUC), Univ Coimbra, Rua Larga, Coimbra, Portugal

J. Adv. Pharm. Technol. Res.

INTRODUCTION

Honeybee venom (HBV) or apitoxin is a biotoxin produced in the venom gland of honeybees (Apidae family), under its abdominal cavity. It has been suggested that HBV represents much more than a classical stereotype of defense against predators, acting also as a medium of social antisepsis contributing to the colony collective immunity. Even without a complete scientific proof of its efficacy/safety, the use in traditional medicine is recurrent for the prevention and treatment of many diseases such as arthritis, rheumatism, pain cancer, skin problems, among others. Moreover, it acts as anti-inflammatory, leishmanicidal agent, antimicrobial, antiviral, antiapoptotic, wound healer, antifibrinolytic and antielastolytic. More recently HBV has been appointed in complementary treatments against SARS-CoV-2.

In this review, a brief information about the HBV compounds and its extraction will be provided before the presentation of the various analytical methods described in the literature. The information discussed within this manuscript was searched using PubMed, Google Scholar, and Science Direct filtering with common terms such as “apitoxin,” “apis,” “bee venom,” “bee products,” and “honey bee.” The documents were analyzed to allow a deeper description on the most

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPedknow_reprints@wolterskluwer.com

How to cite this article: El Mehdi I, Falcão SI, Boujraf S, Mustapha H, Campos MG, Vilas-Boas M. Analytical methods for honeybee venom characterization. J Adv Pharm Technol Res 2022;13:154-60.
relevant compounds present on HBV, considering the number of studies on the literature and the use of different techniques applied in the evaluation.

**HBV COMPONENTS**

HBV compounds have a wide range of pharmacological targets and have been extensively studied as sources for new drugs. An overview has been published on the therapeutic potential and some preclinical trials using HBV. The toxin, with more than 80% of water, is a liquid mixture of active substances covering proteins, peptides, enzymes, and other small molecules. To guarantee the reproducibility of the results during bioactivity assays or clinical trials with HBV, it is crucial to perform the identification of these compounds and standardize the HBV extract.

Melittin, the main component of HBV, is the most studied active membrane protein, consisting of 26 amino acid residues. Its amphiphilic property makes it water soluble and naturally associable with any type of membranes, including artificial. The major neurotoxin in HBV is apamin. This compound is an octadecapeptide that contains four cysteine residues with two disulfide bonds, resulting in stabilization of its tertiary structure for the expression of biological activity.

The mast cell degranulating peptide (MCDP), a 22 residue polypeptide stable over the pH 2-8, presents two disulfide bridges and a terminal amide group. MCDP is not a true anti-inflammatory agent, but employs its activity via the mediation of the anti-inflammatory effect.

Another important compound of HBV is secapin, a polycationic peptide with 25 amino acid residues. It is stabilized by an intramolecular disulfide bridge formed between cysteine residues. Secapin has been shown in two isoforms, secapin-1 and secapin-2 with a similar secondary structure.

Other peptides found in lower quantities in HVB are adolapin and tertiapin. The first is a basic polypeptide with 103 amino acids residues, exhibiting an antinociceptive and anti-inflammatory activity through inhibition of cyclo-oxygenase function. While tertiapin is a presynaptic neurotoxin composed by 21 amino acid residues. The single methionine residue in tertiapin can be readily oxidized, and consequently, its affinity for inhibiting certain types of inward-rectifier K+ channels will be 4-5-fold lower.

The major allergen compound of HBV is phospholipase A2 (PLA2), a glycoprotein. A revised study based on the analysis of the cDNA for PLA2 from HBV gland showed that the sequence of the mature PLA2 comprises 134 amino acids with a single glycosylation site which is cross-linked by four disulfide bridges responsible for their stability and their folding mechanism. PLA2 belongs to the secreted group III sPLA2 enzymes and shows its inflammatory activity by inducing the biosynthesis of prostaglandin, arachidonic, and lysophosphatidic acids.

The last compound highlighted in this review is not involved in the toxic effect but can contribute for the characterization of the product. Hyaluronoglucosaminidase (Hya) plays a role as a spreading factor since the depolymerization and the hydrolysis of the substrate enable the diffusion of the venom constituents. Hya is a glycoprotein with 349 amino acids and four cysteine bridged by two disulfides, with significant similarity with human hyaluronidase, which is tangled in fertilization and the hyaluronan turnover.

The volatile composition of HBV is also a key point in its characterization, since it is directly linked with the social behavior of the insect. Qualitative and quantitative differences were reported depending on the bee species, but also depending on the cast or age within the same species. For instance, isoamyl acetate, absent in queens and newly hatched bee workers, is one of the main components of adult bee sting volatiles. Over than 20 volatile components have been unveiled, which the major of them are (z)-11-eicosen-1-ol, iso-pentyl acetate, n-octyl acetate, benzyl acetate, iso-pentanol, 2-nonanol, n-hexyl acetate, benzyl alcohol, n-butyl acetate, and n-decyl acetate.
Several metal elements were also reported in HBV including K, Na, Ca, a, Mg, Cu, Zn, B, Al, V, Mn, Co, Ni, Sr, and Mo, but also toxic metals such as As, Ba, Cd, Sb, Cr, and Pb. Its content was suggested to be dependent on floral species and season, but its quantities should not surpass the levels established on pharmacopeias. The identification of metal contamination, mainly the most toxic, is highly recommended to guarantee the safety, conformity, and quality of HBV as a pharmaceutical raw material. Unfortunately, there is a noteworthy insufficiency of data in literature concerning metal contamination in this product.

**HONEYBEE VENOM COLLECTION**

Collecting fresh HBV requires careful work with the highest degree of cleanliness. From a single bee, the collection of venom can be performed using a glass capillary and gently squeezing the venom sac or, alternatively, the sac and gland can be dissected and opened by repeatedly puncturing with a sharp needle. For collecting HBV on a commercial scale, several collector devices, based on the stimulating of the bees with electrical current pulses, have been built. The first, was built on a wood frame crossed by metallic wires, with an inner glass plate covered tightly with a nylon sheet, and subject to a specific voltage as a stimulating tension. However, further developments allowed to optimize the position of venom-collecting frames in beehive, and the coupling of stimulating factors: electrical and sound stimulus.

**HONEYBEE VENOM CHARACTERIZATION: ANALYTICAL TECHNIQUES**

Several methods have been applied in bee venom characterization, which include biological tests, chemical approaches based on typical protein reaction, and separation techniques. The choice may depend on several factors, particularly the non-proportionality of HBV components: the high level of melittin and PLA2 may mask the detection of minor compounds. Unfortunately, there are still no methods that allow product standardization, and the purification stage of HBV components remains a problem, due to the combinations of factors such as the highly alkaline profile, the similar molecular weight of the most relevant compounds, and the mixture complexity. Table 1 resumes the analytical methods used to access the main components.

**Table 1: Analytical techniques to access honeybee venom components**

| Type          | Technique                        | HBV components                  | References        |
|---------------|----------------------------------|---------------------------------|-------------------|
| Biochemical   | Enzymatic                        |                                 |                   |
|               | Phospholipase assay              | PLA2                            | [26,32,33]        |
|               | Melittin assay                   | Melittin                        | [26]              |
|               | Allergosorbent assay             | PLA2                            | [25]              |
| Electrophoresis| SDS-PAGE                         | PLA2; melittin                  | [34]              |
|               | Propionate acid/urea PAGE        | PLA2; melittin; MCDP            | [35]              |
|               | Isoelectric focusing             | PLA2; melittin                  | [26]              |
| Chromatography| HPLC                             | PLA2; hyaluronidase; melittin; apamin; MCDP | [36,37]          |
|               | HPLC-MS                          | Melittin; apamin; MCDP; secapin; histamine; free amino acid; sugars, PLA2 | [31,38]          |
|               | GC-MS                            | Volatiles                       | [9,20]            |
| Spectroscopy  | Liquid scintillation counting    | PLA2                            | [32]              |
|               | Fluorescence                     | Melittin; apamin; MCDP          | [38]              |
|               | Infrared                         | Biological active components    | [37,39]           |
|               | ICP-MS                           | Metals                          | [24]              |
| Electrochemistry| Stripping voltammetry           | Melittin                         | [40,41]           |
|               |                                  | Metals                          |                   |

HBV: Honeybee venom, PAGE: Polyacrylamide gel electrophoresis, SDS-PAGE: Sodium dodecyl sulfate-PAGE, HPLC: High-performance liquid chromatography, MS: Mass spectrometry, GC-MS: Gas chromatography-MS, ICP-MS: Inductively coupled plasma-MS, PLA2: Phospholipase A2, MCDP: Mast cell degranulating peptide
Capillary electrophoresis is another option for the determination of melittin. The separation occurs in a double capillary system with UV-detection at two different wavelengths.\[43\] The output revealed a good performance for systematic analysis of HBV.

Despite this, HPLC is the main chromatographic technique used for peptides and proteins, either for quality control purposes, fingerprinting, or standardization of the product [Table 2]. Exclusion HPLC (SEC-HPLC) under isocratic conditions can identify melittin, apamin, and MCDP; nevertheless, due to melittin/apamin ratio (30:1) and the chemical behavior, it additionally requires reverse phase with C18 columns (RP-18) to determine apamin alone, implying quantification of melittin by subtraction.\[33\] Different modifications to improve these methods can be found in the literature, such as the use of RP-18 preparative columns, addition of cytochrome c as internal standard, or coupling with intrinsic fluorescence detection (IFD).\[36,38,44,45\]

Spectrometry has become the key tool for HBV analysis. Multiple platforms with distinct capabilities were explored, such as matrix-assisted laser desorption/ionization with time-of-flight detector (MALDI-TOF), liquid chromatography with electrospray ionization detector coupled to mass spectrometry (LC-ESI/MS), ion trap instruments, and mass spectrometry with Fourier transform systems mass spectrometry (FT-MS).\[18,46,47\] Melittin and apamin were simultaneously quantified through their precursor ions \([M + 5H]^{5+} (m/z 570.2)\) and \([M + 4H]^{4+} (m/z 507.7)\), respectively, using a triple quadrupole tandem mass spectrometer combined with an ESI interface with multiple reaction monitoring.\[10\] The combination of two complementary mass spectrometry platforms, MALDI-TOF-MS and nano-electrospray ionization quadrupole TOF MS (nanoESI-QqTOF-MS), enabled the development of a precise and accurate HBV characterization process, using an internal standard and two different matrices, sinapinic acid and \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA): apamin and MCDP were detected with CHCA but not with sinapinic acid, which instead enabled the detection of four degradation products of melittin.\[40\]

The spectroscopic techniques have been used mainly for melittin, apamin, and tertiapin structural analysis, which involve peptides in solution at different temperature conditions. The three-dimensional structure of melittin was detected by X-ray crystallography, indicating a tetramer holding at least 2-fold axis of rotation. Proton nuclear magnetic resonance spectroscopy (\(^1\)HNMR) was also employed to understand melittin behavior at different temperature, pH, and ionic strength confirming an amphiphilic spatial structure, which is stabilized by forming mixed micelles in ionic solution or self-aggregation.\[49\] Two-dimensional nuclear magnetic resonance spectroscopy was also useful for evaluation of the apamin structure, enabling the observation of an \(\alpha\)-helical core with fraying residues, and a \(\beta\)-turn.\[50\] However, some differences where reported when comparing with the outputs of distance geometry derived from NMR.\[51\]

Electrochemical methods were also used for HBV characterization and its introduction was achieved exploring proportionality between the decrease on oxidation current of ferrocene at 110 mV and the increase of melittin concentration. This indirect activity, measured by square wave stripping voltammetry on gold electrodes, using nonaqueous solvents as dimethyl sulfoxide and acetoniitrile, proved to be a fast and low cost approach, avoiding preconcentration procedures.\[40\]

**Enzymes**

The allergenic effect of HBV is due to the presence of enzymes, with fifty five already described, including PLA2, phospholipase B, hyaluronidase, phosphatase acid, and \(\alpha\)-glucosidase.\[17,52\] In this review, we focus on the major, PLA2 and hyaluronidase.

---

**Table 2: Chromatography conditions used for honeybee venom peptides/enzymes identification**

| Chromatography technique | Column type and elution conditions | Detection | Components | References |
|--------------------------|-----------------------------------|-----------|------------|-----------|
| SEC-HPLC                 | I-125/isocratic                    | DAD       | Apamin, hya, MCDP, melittin, PLA2 | [33] |
| RP-HPLC                  | LiChrospher Si100, RP-18/isocratic | DAD       | Apamin, hya, MCDP, melittin, PLA2 | [36] |
| RP-HPLC                  | Hypersil WP-300                    | UVD       | Apamin, hya, MCDP, melittin, PLA2, procamine, tertiapin, secapin | [36] |
| RP-HPLC                  | C18 (100/180/300Å)/linear gradient | UVD       | Apamin, melittin, PLA2 | [46] |
| RP-HPLC                  | Synchropack C8/linear gradient     | DAD       | Apamin, MCDP, melittin, PLA2 | [45] |
| RP-HPLC                  | Sepax Bio-C18                      | IFD       | Melittin | [38] |
| RP-HPLC                  | XSelect CSH130 C18/linear gradient | DAD       | Apamin, melittin, PLA2 | [47] |

PLA2: Phospholipase A2, MCDP: Mast cell degranulating peptide, DAD: Diode array detection, IFD: Intrinsic fluorescence detection, UVD: Ultraviolet detector, RP-18: Reverse phase with C18 columns, HPLC: High-performance liquid chromatography, SEC-HPLC: Size exclusion chromatography-HPLC, RP-HPLC: Reversed phase-HPLC.
The first attempt on fractioning PLA2 and hyaluronidase from HBV was made with dialysis membranes using cellophane tubing and gel filtration on sephadex columns. This procedure allowed the majority of the HBV components to pass through the column, but retaining hyaluronidase and partially PLA2.[52] The use of a stepped-gradient open column with ethanol/water elution, proved also to be adequate for the purification and further quantification of PLA2.[53]

To analyze PLA2 and its isoforms, electrophoresis is a common methodology either using propionic acid/urea gels, thin-layer isoelectric focusing polyacrylamide gels, or sodium dodecyl sulfate polyacrylamide gels.[26,35] The combination of capillary electrophoresis with UV-DAD proved to be a suitable tool for monitoring PLA2 and assessing traceability and authenticity of HBV.[43]

The characterization of HBV enzymes using chromatography techniques is also recurrent, and most of the protocols applied for peptide analysis are frequently optimized for PLA2 [Table 2].[53,44,45] For hyaluronidase, exclusion chromatography on a I-125 protein column, under isocratic conditions, enabled its identification at 200–230 nm,[33] however, a better performance was achieved when using DeltaPak, which reduces, specifically, the elution time of hyaluronidase due to its interaction with the hydrophobic stationary phase.[56]

ESI-MS,[6], nanoESI-MS, and ESI-QTOF-MS are among the many different spectrometric methods explore for the evaluation of HBV enzymes.[67] NanoHPLC-nanoESI-MS was used to evaluate PLA2 production at particular climatic conditions, while MALDI mass spectrometry imaging was applied to map the diffusion and distribution of PLA2 over time.[53,54] Electrospray ionization linear quadrupole FT ion cyclotron resonance (ESI-LTQ-FT-ICR), MALDI-TOF/TOF, ESI-QTOF, can also be used for characterization of hyaluronidase.[8,44]

The secondary and tertiary structural information on PLA2 has been revealed using X-ray diffraction, molecular dynamics and NMR spectroscopic techniques,[65] whereas its primary structure was identified by tandem MS and bioinformatics, exploring the protein sequence derived from cDNA and mRNA.[50] X-ray crystallography was also employed to evaluate the crystal structure of hyaluronidase oligomer revealing more than 50% sequence identity with hyaluronidase from other hymenoptera.[19]

Volatiles

Three techniques have been explored to collect the HBV volatile fraction, either recovering the volatile compounds from the whole sting apparatus,[57] using a solid phase microextraction fiber to collect the volatiles from the airspace around caged bees[20] or the collection of the volatile fraction while bees are stinging.[9] The last one requires a dry air pulsating device which creates an airflow carrying away the HBV volatile components while bees are sting and pierce a porous membrane. Subsequently, by condensation at ~90°C, the volatiles are brought into solution and analyzed by GC-MS.[19] Results produced showed quantitative and qualitative differences of volatile substances either in stings or in fresh HBV. This variation is related to the denaturation process which immediately triggers after the extrusion of HBV next stinging or extraction. Over than 20 volatile components have been unveiled from HBV, which the most significant beeing the (z)-11-eicosen-1-ol, Figure 2, and the esters fraction, higly represented by the isoamyl acetate.[9,20-22,57]

Metals and minerals

Metal content monitoring in HBV is an extremely important issue; nonetheless, the literature information on this topic is limited.[24,43] Inductively Coupled Plasma MS (ICP-MS) was applied in the evaluation of twenty contaminant elements, revealing the year to year variation. In this work, the toxic metals levels in HBV were below the permissible for drug substances.[24] A more recent approach for the evaluation of heavy metals in HBV explored the anodic stripping voltammetry using either mercury drop electrode or PLA/carbon filament after a previous wet pressure digestion to enable sample dissolution. The results between the two electrodes were equivalent but with the mercury drop exhibiting higher sensitivity and lower detection limits.[41]

Sugars

Structural resemblances, hydrophilic characteristics, low proton affinity, and ionizability of the oligosaccharides make their characterization tricky. In HBV, they are frequently analyzed in the context of protein-linked sugars. N-linked oligosaccharides of PLA2 and hyaluronidase were the most investigated using GC after hydrolysis of the glycoprotein/glycopeptide in methanolic hydrogen chloride.[58] Glucose, fructose, and vanilloloside were identified and quantified in HBV using LC-Q-Orbitrap-MS untargeted metabolomics analysis.[59] while mannose, galactose, fructose, and N-acetylglucosamine were the main PLA2-linked monosaccharides detected after hydrolysis by an endoglycosidase-H.[60]

Free amino acids

Amino acids constitute the major precursors of HBV catecholamines, peptides, and proteins biosynthesis.[61] Gel filtration, paper, and thin-layer chromatography are used to estimate HBV-free amino acids (less than 1%), and among them, arginine, cystine, glutamic acid, and histidine are the most reported.[62] Others, such as aspartate, glutamate, serine, alanine, glycine, phenyl-ethanolamine, gamma-aminobutyric acid, and tyrosine, can also be detected using RP-HPLC and electrochemical detection.[62]
CONCLUSION AND FUTURE PERSPECTIVES

The interest on bee venom research is increasing due to its potential therapeutic use as anti-inflammatory, antimicrobial, or cytotoxicity. The complexity of its composition demands a constant development on methodologies and techniques that may fraction and detect the different constituents enabling to identify its biopotential at low risk. This review highlights the different techniques and analysis which have been used to characterize this matrix. Various enzymatic, electroanalytical, spectroscopic, and spectrometric techniques can be applied for the standardization of the product and inference on its content. Those techniques allow the separation, purification, quantification, chemical, and structural determination of the biocompounds, adding important quality value to the product, proving its feasibility in quality control and specific fingerprinting. Moreover, coupled methods and big data engineering undone a new task to identify the whole product proteome and therefore set up a standardization approach, to design good manufacturing practices of pharmaceutical products.

Financial support and sponsorship

Thanks to the Foundation for Science and Technology (FCT, Portugal) for support by national funds to CIMO (UIDB/00690/2020). The project PDR2020-1.0.1-FEADER-031734: “DivInA-Diversification and Innovation on Beekeeping Production” and to the European Regional Development Fund through the Regional Operational Program North 2020, within the scope of Project GreenHealth, Norte-01-0145-FEDER-000042. This research was also funded by (UI0204): UIDB/00313/2020, Center of Chemistry from Faculty of Sciences and Technology of University of Coimbra, Portugal.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Rady I, Siddiqui IA, Rady M, Mukhtar H. Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer Lett 2017;402:16-31.
2. Baracchi D, Francese S, Turillazzi S. Beyond the antipredatory defence: Honey bee venom function as a component of social immunity. Toxicicon 2011;58:550-7.
3. Kasozi KI, Niedbała G, Alqarni M, Zirintunda G, Ssempijja F, et al. Bee venom-A potential complementary medicine candidate for SARS-CoV-2 infections. Front Public Health 2020;8:594458.
4. Abd El-Wahed AA, Khalifa SA, Sheikh BY, Farag MA, Saeed A, Larik FA, et al. Bee venom composition: From chemistry to biological activity. Stud Nat Prod Chem 2018;60:459-84.
5. Kocygilt A, Metin E, Kalem S. Anti-inflammatory and antioxidative properties of honey bee venom on Freund’s Complete Adjuvant-induced arthritis model in rats. Toxicicon 2019;161:4-11.
6.WEBHE R, Frangièh J, Rima M, EI Obeid D, Sabatier JM, Fajloun Z. Bee venom: Overview of main compounds and bioactivities for therapeutic interests. Molecules 2019;24:2997.
7. Lin TY, Hsieh CL. Clinical applications of bee venom acupuncture injection. Toxins (Basel) 2020;12:618.
8. Matsyiak J, Hajduk J, Mayer F, Hebeler R, Kokot ZJ. Hyphenated LC-MALDI-ToF/ToF and LC-ESI-QToF approach in proteomic characterization of honeybee venom. J Pharm Biomed Anal 2016;121:69-76.
9. Domerego R. La thérapie au venin d’abeille. Chaudon-Norante: Baroch editions; 2012.
10. Zhou J, Zhao J, Zhang S, Shen J, Qi Y, Xue X, et al. Quantification of melittin and amin in bee venom lyophilized powder from Apis mellifera by liquid chromatography-diode array detector-tandem mass spectrometry. Anal Biochem 2010;404:171-8.
11. Banks BE, Dempsey CE, Vernon CA, Warner JA, Yamey J. Anti-inflammatory activity of bee venom peptide 401 (mast cell degranulating peptide) and compound 48/80 results from mast cell degranulation in vivo. Br J Pharmacol 1990;99:350-4.
12. Matsyiak J, Schmelzer CE, Neubert RH, Kokot ZJ. Characterization of honeybee venom by MALDI-TOF and nanoESI-QToF mass spectrometry. J Pharm Biomed Anal 2011;54:273-8.
13. Dos Santos-Pinto JR, Perez-Riverol A, Lasa AM, Palma MS. Diversity of peptidic and proteinaceous toxins from social Hymenoptera venoms. Toxicon 2018;148:172-96.
14. Mourelle D, Brigatte P, Bringanti LD, De Souza BM, Arcuri HA, Gomes PC, et al. Hyperalgesic and edematogenic effects of Secapin-2, a peptide isolated from Africanized honeybee (Apis mellifera) venom. Peptides 2014;59:42-52.
15. Chen J, Lariviere WR. The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword. Prog Neurobiol 2010;92:151-83.
16. Doupnik CA. Venom-derived peptides inhibiting Kir channels: Past, present, and future. Neuropharmacology 2017;127:161-72.
17. Hossen MS, Shapla UM, Gan SH, Khalil MI. Impact of bee venom enzymes on diseases and immune responses. Molecules 2016;22:25.
18. Gmachl M, Kreil G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. Proc Natl Acad Sci U S A 1993;90:3569-73.
19. Marković-Housley Z, Miglierini G, Soldatova L, Rizkallah PJ, Müller U, Schirmer T. Crystal structure of hyaluronidase, a major component of bee venom, and its conjugates in cancer therapy. J Exp Biol 2014;217:3512-8.
20. Shoham Y, Eshkol S, Reisman RE, Brenner A, Barshack I. Identification of isoamyl acetate as an active component in the sting pheromone of the honey bee. Nature 1962;195:1018-20.
21. Pickett JA, Williams IH, Martin AP. (Z)-11-eicosen-1-ol, an important new pheromonal component from the sting of the honey bee, Apis mellifera L. (Hymenoptera, Apidae.). J Chem Ecol 1982;8:163-75.
22. Hider RC. Honeybee venom. Bee World 1989;70:55-76.
23. Kokot ZJ, Matsyiak J. Inductively coupled plasma mass spectrometry determination of metals in honeybee venom. J Pharm Biomed Anal 2008;48:955-9.
24. Owen MD, Pfaff LA, Reisman RE, Wypych J. Phospholipase A2 in venom extracts from honey bees (Apis mellifera L.) of different ages. Toxicon 1990;28:813-20.
25. Schumacher MJ, Schmidt JO, Egen NB, Dillon KA. Biochemical variability of venoms from individual European and Africanized honeybees (Apis mellifera). J Allergy Clin Immunol 1992;90:59-65.
27. De Graaf DC, Regina M, Braga B, Magalhães RM, Abreu D, Blank S, et al. Standard methods for *Apis mellifera* venom research. J Apic Res 2021;60:1-31.

28. Benton AW, Morse RA, Stewart JD. Venom collection from honey bees. Science 1963;142:228-30.

29. Michal Rybak PS. Application of coupled electrical and sound stimulation for honeybee venom collection. J Apic Sci 2007;51:69-6.

30. Lee Y, Kim SG, Kim IS, Lee HD. Standardization of the manufacturing process of bee venom pharmacopuncture containing melittin as the active ingredient. Evid Based Complement Alternat Med 2018;2018:2353280.

31. Scaccabarozzi D, Dods K, Le TT, Guummer JP, Lussu M, Milne L, et al. Factors driving the compositional diversity of *Apis mellifera* bee venom from a *Corymbia calophylla* (marr) ecosystem, Southwestern Australia. PLoS One 2021;16:e0253838.

32. Marz R, Mollay C, Krei G, Zelger J. Queen bee venom contains much less phospholipase than worker bee venom. Insect Biochem 1981;11:685-90.

33. Räder K, Wildfleuter A, Wintersberger F, Bossinger P, Mücke HW. Characterization of bee venom and its main components by high-performance liquid chromatography. J Chromatogr 1987;408:341-8.

34. Pacáková V, Štulík K, Thi Hau P, Jelínek I, Vinš I, Sýkora D. Liquid chromatographic analysis and separation of polypeptide components from honey bee venom. J Liq Chromatogr 1994;17:3333-49.

35. Chettibi S, Lawrence A. High resolution of honey bee (*Apis mellifera*) venom peptides by propionic acid/urea polyacrylamide gel electrophoresis after ethanol precipitation. Toxicon 1989;27:781-7.

36. Szókán GY, Horváth J, Almás M, Sáftsics GY, Palócz A. Liquid chromatographic analysis and separation of polypeptide components from honey bee venom. J Liq Chromatogr 1994;17:3333-49.

37. Tanuwidjaja I, Sichevjak L, Gugid C, Levaníc M, Jurić S, Vinceković M, et al. Chemical profiling and antimicrobial properties of honey bee (*Apis mellifera L.*) venoms. Molecules 2021;26:3049.

38. Dong J, Ying B, Huang S, Ma S, Long P, Tu X, et al. High-performance liquid chromatography combined with intrinsic fluorescence detection to analyse melittin in individual honeybee (*Apis mellifera*) venom sac. J Chromatogr B Analyt Technol Biomed Life Sci 2015;1002:139-43.

39. Nasser M. The potential of natural venom of *Apis mellifera* for the control of grains weevil adults (sitophilus granarius-coleopter). Int J Entomol Res 2013;1:25-31.

40. Yilmaz UT, Melekgollurli T, Kekillioglu A, Uzun D. Sensitive voltammetric determination of melittin in honeybee venom powder from *Apis mellifera*. Microchem J 2016;124:364-7.

41. Choinski M, Hrdlička V, Šestáková I, Navrátil T. Voltammetric determination of heavy metals in honey bee venom using hanging mercury drop electrode and PLA/carbon conductive filament for 3D printer. Monats Chem 2021;152:35-41.

42. Banks BE, Dempsey CE, Pearce FL, Vernon CA, Wholley TE. New methods of isolating bee venom peptides. Anal Biochem 1981;116:48-52.

43. Kokot ZJ, Matusiak J, Urbaniai B, Derezinski P, New CZE-DAD method for honeybee venom analysis and standardization of the product. Anal Bioanal Chem 2011;399:2487-94.

44. Kokot ZJ, Matusiak J. Simultaneous determination of major constituents of honeybee venom by LC-DAD. Chromatographia 2009;69:1401-5.

45. Rybak-Chmielewska H, Szczesna T. HPLC study of chemical composition of honeybee (*Apis mellifera L.*) venom. J Apic Sci 2004;48:103-9.

46. Sobral F, Sampaio A, Falcão S, Queirós MJ, Calhelha RC, Vilas-Boas M, et al. Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. Food Chem Toxicol 2016;94:172-7.

47. Frangieh J, Salma Y, Haddad K, Mattel C, Legros C, Fajloun Z, et al. First characterization of the venom from *Apis melifera syriaca*, a honeybee from the Middle East region. Toxins (Basel) 2019;11:191.

48. Matysiak J, Hajduk J, Pietrzak L, Schmelzer CE, Kokot ZJ. Shotgun proteome analysis of honeybee venom using targeted enrichment strategies. Toxicon 2014;90:255-64.

49. Brown LR, Lauterwein J, Wüthrich K. High-resolution 1H-NMR studies of self-aggregation of melittin in aqueous solution. Biochim Biophys Acta 1980;622:231-44.

50. Wemmer D, Kallenbach NR. Structure of apamin in solution: A two-dimensional nuclear magnetic resonance study. Biochemistry 1983;22:1901-6.

51. Pease JH, Wemmer DE. Solution structure of apamin determined by nuclear magnetic resonance and distance geometry. Biochemistry 1988;27:8491-8.

52. Franklin R, Baer H. Comparison of honeybee venoms and their components from various sources. J Allergy Clin Immunol 1975;55:285-98.

53. Ferreira Junior RS, Sciani JM, Marques-Porto R, Junior AL, Orsi RdeO, Barraviera B, et al. Africanized honey bee (*Apis mellifera*) venom profiling: Seasonal variation of melittin and phospholipase A (2) levels. Toxicon 2010;56:355-62.

54. Frances S, Lambard D, Mastrobuon G, La Marca G, Moneti G, Turillazzi S. Detection of honeybee venom in envenomed tissues by direct MALDI MSI. J Am Soc Mass Spectrom 2009;20:112-23.

55. Arni RK, Ward RJ. Phospholipase A2 – A structural review. Toxicon 1996;34:827-41.

56. Saraswathy N, Ramalingam P, Saraswathy N, Ramalingam P. Protein sequencing techniques. In: Concepts Tech Genomics Proteomics. Amsterdam: Elsevier; 2011. p. 193-201.

57. Lakshmi Priya G, Rameshkumar P, Ponnamickam P. Identification of volatile and protein profiles in the sting and mandibular glands of the worker honey bee (*Apis cerana indica*). Biochem Physiol 2013;2:1-7.

58. Shipolini RA, Callawaert GL, Cottrell RC, Vernon CA. The amino-acid sequence and carbohydrate content of phospholipase A2 from bee venom. Eur J Biochem 1974;48:465-76.

59. Kluczenysa A, Pleva S, Derezinski P, Garrett TJ, Rubio VY, Kokot ZJ, et al. Identification and quantification of honeybee venom constituents by multiplatform metabolomics. Nat Res 2020;10:1-11.

60. Weber A, März L, Altmann F. Characteristics of the asparagine-linked oligosaccharide from honey-bee venom phospholipase A2. Evidence for the presence of terminal N-acetylglucosamine and fucose in an insect glycoprotein. Comp Biochem Physiol B 1986;83:321-4.

61. Surendra SS, Ravikumar H, Reddy MR. Evaluation of catecholamines and amino acids from venom reservoir extract of Indian honey bee (*Apis*) species. J Apis Res 2014;53:514-9.

62. Nelson DA, O’Connor R. The venom of the honeybee (*Apis mellifera*): Free amino acids and peptides. Can J Biochem 1968;46:1221-6.