EL Mehdi Iouraouine

EVALUATION OF THERAPEUTIC INTERESTS OF BEE VENOM FROM *APIS MELLIFERA INTERMISSA* AS A BIOACTIVE INGREDIENT IN MELANOMAS

UNIVERSIDADE D COIMBRA

1 2 9 0

1 2 9 0

UNIVERSIDADE D
COIMBRA

جامعة سيدي محمد بن عبد الله – فاس

Université Sidi Mohamed Ben Abdellah - Fès

EL Mehdi Iouraouine

EVALUATION OF THERAPEUTIC INTERESTS OF BEE VENOM FROM *APIS MELLIFERA INTERMISSA* AS A BIOACTIVE INGREDIENT IN MELANOMAS

Ph.D. thesis in cotutelle
between
University of Coimbra, Portugal, and Sidi Mohamed Ben Abdellah University
Fez, Morocco.
Ph.D. thesis submitted to the pharmaceutical sciences, supervised by Professor Maria G. Campos and Professor Mustapha Harandou, and presented in the Faculty of Sciences and Techniques of Sidi Mohamed Ben Abdellah University.

March 2021

جامعة سيدي محمد بن عبد الله – فاس
Université Sidi Mohamed Ben Abdellah - Fès

1 2 UNIVERSIDADE D 9 0
COIMBRA

Order number: /2020

Doctorate thesis

Presented by

Mr : El Mehdi IOURAOUINE

Specialty: Pharmaceutical sciences

Thesis subject: Evaluation of Therapeutic Interests of Bee Venom from *Apis mellifera intermissa* as a Bioactive Ingredient in Melanomas

A thesis presented and defended on March 31st, 2021, before the jury composed of:

Prof. Saïd Boujraf	Sidi Mohamed Ben Abdellah, Fez University	President
Prof. Abdelkader Dahchour	Sidi Mohamed Ben Abdellah, Fez University	Reporter
Prof. Omar `Senhaji	Moulay Ismail, Meknes University	Reporter
Prof. M. A. Elamrani	Abdelmalek Essaadi, Tetouan University	Reporter
Prof. Samira Rabhi	Sidi Mohamed Ben Abdellah, Fez University	Reviewer
Prof. Mohamed Chikri	Sidi Mohamed Ben Abdellah, Fez University	Reviewer
Prof. Mustapha Harandou	Sidi Mohamed Ben Abdellah, Fez University	Advisor
Prof. Maria G. Campos	Coimbra University, Portugal	Advisor

Host laboratory: Clinical Neurosciences Laboratory-Biophysics Laboratory and Clinical IRM Methods

1 Acknowledgments:

First and foremost, I would like to express my genuine gratitude to my supervisors Prof. Mustapha Harandou and Prof. Maria Graça Campos. They allowed me to do research and provided me with invaluable guidance and continuous support to make this experience productive and fruitful. It is an honor to work with professors whose dynamism and motivation have deeply inspired me.

I also wish to sincerely thank Prof. Miguel Vilas-Boas for his priceless advice, and insightful comments, Prof. Ofélia Anjos for her valuable help and beneficial information, and Dr. Soraia Isabel Domingues Marcos Falcão, whose assistance has been advantageous, without forgetting Dr. Manuel Ricardo Costa Calhelha, and David João Teixeira Alves Cabral to whom I send my warmest regards. All those people contributed greatly to the improvement of this thesis and improved my luggage as a researcher.

This work has highly benefited from the cooperation of the IPB/The Mountain Research Centre (CIMO), particularly Prof. Isabel C.F.R. Ferreira, who I appreciate even if I have not had the chance to meet her in person, and the FMPF/Clinical Neurosciences Laboratory Department of Biophysics and Clinical MRI Methods, in the name of Prof. Said Boujraf to whom I am deeply indebted for always looking after me.

To the people who let me work with their precious bees Mr. Lahcen Messaoudi; may God have mercy on him, Mr. El Hassan Idouissaadan, Mr. Mohamed Benchaikh, Mr. Mohamed Sahraoui, and our ingenious Yahya Messaoudi, thanks a million, the research would have been much tricky without your aid and your kindness.

I am deeply grateful to my colleagues and those I enjoyed working with during this project and other related ones.

2 Dedications:

To my father and mother,

Nobody has been leading me in the pursuit of this project other than my family members. I want to thank my parents, whom I wished were with us today, but Allah's will have decided otherwise, but whose love and blessings have guided and taken care of me all the time. It is your education that made me never stop seeking knowledge and hoping to make you proud.

To my wife,

I wish to declare my thankfulness to my loving and supporting wife, Lalla Latifa, who had faith in me since the beginning and was so patient during this process. I am so lucky to have such a fighter beside me.

To my children,

Lalla Fatima Zahraa, Lalla Aya, and Sidi Mohammad Ismaël, my little treasures that provide unending inspiration, transferred their positive energy, laughter, and comfort and gave me the power to strive even more.

To my sisters, brothers, and my dear aunt

I appreciate your honest wishes and heartful prayers, and I wish you and your families a blessed successful life.

To my friends,

Thank you for your thoughts, calls, visits, and for being there and offering help even when I did not ask for it.

To the soul of my beloved and unforgettable brother Zakaria…

3 Index of figure :

4 Index of tables :

5 Abbreviations:

μHPLC:	micro–High-Performance Liquid Chromatography.
^{1}HNMR:	Proton Nuclear Magnetic Resonance.
4EBP1	Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1.
ADAMTS-5	A disinterring and metalloproteinase with thrombospondin. motif-5.
Akt	Protein kinase B.
ATF-6α	Activating transcription factor 6 alpha.
ATF-6β	Activating transcription factor 6 beta.
BRAF	BRAF is known as a "switch gene." It produces a protein that normally regulates skin cells, allowing them to multiply only when needed. But genetic changes (mutations) to BRAF can keep it switched on abnormally, driving out-of-control tumor growth. About half of all melanoma patients have the BRAF mutation. Targeted therapies have significantly improved outcomes for patients with advanced melanoma who have the BRAF mutation.
BVG:	Bee venom gland.
CD:	Circular dichroism spectroscopy.
cDNA:	Complementary DNA.
CE:	Capillary electrophoresis.

CHCA:	Sinapinic acid α-cyano-4-hydroxycinnamic acid.
CPLL:	Combinatorial peptide ligand library.
CZE:	Capillary zone electrophoresis.
DAD:	Diode-array-detector.
DBV:	Dried bee venom.
DMPC:	1,2-Dimyristoyl-sn-glycero-3-phosphocholine.
DMSO:	Dimethyl sulfoxide.
DR	Death receptor
EDQM:	European Directorate for the Quality of Medicines.
EGF	Epidermal growth factor.
EGFR	Epidermal growth factor receptor.
ESI-MS/MS:	Electrospray ionization tandem mass spectroscopy.
F-actin	Filamentous-actin.
FAK	Focal adhesion kinase.
Fas	Also known as CD95, is a cysteine-rich transmembrane protein, which is expressed on the cells of the lymphoid system, heart, and in various places throughout embryonic and adult life.
FasL	Fas ligand.
FCHE:	Fluorophore-assisted carbohydrate electrophoresis.

FT-MS:	Fourier transform mass spectroscopy.
GADD153	Growth arrest- and DNA damage-inducible gene 153
GBP	Good beekeeping practicees.
GC-MS:	Gas chromatography-mass spectrometry.
GRP78	Is also called binding immunoglobulin protein (BiP).
GSTS1:	Glutathione-S-transferase sigma1 isoform A.
HBV:	Honey bee venom.
Hya:	Hyaluronidase.
ICP-MS:	Inductively coupled plasma mass spectrometry.
IFD:	Intrinsic fluorescence detection.
IRE-1α	Serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α.
IRE-1α	Serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α.
IS:	Internal standard, shotgun proteomic approaches.
IκBα	NF-κB inhibitor.
JAK2	Janus kinase 2 (non-receptor tyrosine kinase).
JNK	c-Jun N-terminal kinases.
LC-ESI/MS^n	Liquid chromatography-electrospray tandem mass spectroscopy

LC-QqQ-MS/MS	Liquid chromatography-triple quadrupole mass spectrometry.
m/z:	Mass to charge.
MALDI-TOF:	Matrix-assisted laser ionization-time of flight.
MAPK	Mitogen-activated protein kinas.
MCDP:	Mast cell degranulating peptide.
MeCN:	Acetonitrile.
MEK	Mitogen-activated protein kinase kinase (also known as MAP2K, MEK, MAPKK).
Mlt	Melittin.
MMP-9	matrix metalloproteinase-9.
MNL	Mononuclear leucocyte
MPO	Myeloperoxidase
MRJP:	Major royal jolly proteins.
MRM:	Multiple reaction monitoring.
MSC:	Melanoma Skin Cancer.
mTOR	mechanistic target of rapamycin.
mTORC1	Mechanistic target of rapamycin complex 1.
mTORC2	Mechanistic target of rapamycin complex 2.

nanoESI-QqTOF-MS:	Nano-electrospray ionization quadrupole time of flight mass spectroscopy.
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells.
OSI	Oxidative stress index.
PARP	Poly (ADP-ribose) polymerase.
PDBe:	Protein Data Bank Europe,
PDBj:	Protein Data Bank Japan,
PDBsum:	Structural summaries of Proteins Data Bank,
PERK	Protein kinase R-like endoplasmic reticulum kinase.
Pick	Phosphatidylinositol 3-kinase,
PI3K	Phosphatidylinositol-3-kinase.
PLA2:	Phospholipase A2.
PMA	Phorbol 12-myristate 13-acetate
PXR2540:	Peroxiredoxin 2540.
Rab	Rab subfamily is a member of the Ras superfamily of small G-proteins involved in membrane trafficking.
Ras	Subfamily is a member of the Ras superfamily of small G-proteins involved in cell proliferation.

RCSB PDB:	Research Collaboratory for Structural Bioinformatics Proteins Data Bank.
RP-HPLC:	Reverse-phase high-performance liquid chromatography.
RTKs	Receptor tyrosine kinases
S6K1	Ribosomal protein S6 kinase beta-1.
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
SEC-HPLC:	Size exclusion chromatography-high performance liquid chromatography.
SOD1:	Superoxide dismutase.
SOS	Son of Sevenless.
STAT-3	Signal transducer and activator of transcription-3.
SWSV:	Square wave striping voltammetry.
TAS	Total antioxidant status.
TBATFB:	Tetrabutylammonium tetrafluoroborate.
TGF-β cytokines	Transforming growth factor-beta cytokines.
TOS	Total oxidant status.
TPX1:	Thioredoxin peroxidase 1 isoform A.

TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand.
UVD:	Ultraviolet detector.
Δψm	Mitochondrial membrane potential.

6 Abstract:

The ethnopharmacological data from Morocco show strong traditional use of natural tissue and extracts by our ancestors for preventive and curative purposes, representing a valuable and promising guide for research. Furthermore, natural heritage represents an extreme biodiversity richness in bioactive substances. It is the case, for instance, with bee products such as honey, propolis, bee wax, and bee venom. Manuscripts from pharaonic civilization show that ancient Egyptians mummified their dead with honey and other bee products. In Muslim culture, we can easily notice the part of honey in everyday use, and this is related to a whole chapter of the Holy Qur'an, untitled "the bees" (Surat An'Nahl). Healing by bee stings is well known in Eastern cultures as well as in ancient Europe. In line with this, the current project proposal attempts to highlight and evaluate bee venom as a possible bioactive natural substance and therefore value the product as a drug itself or a therapeutic adjuvant. Thence, we claim that its production is a substantial and strategic challenge of sustainable development. This work's main aim was to study the chemical characterization and evaluate "in fin" the potential value of biological activities, essentially the anticancer activity of *Apis mellifera intermissa* venom, the main abundant honey bee species in Morocco. This work followed a straightforward procedure that the strategic key is the improvement of a bee venom collector. We applied a patent request for this device. This improvement affects several levels, including the purity of bee venom, the yield of the collection, the beehives' stability in the apiary, and the decrease of bee damage per collection period. Moreover, this collector provides an affordable device for Moroccan beekeepers. Fifteen *Apis mellifera* venom samples from three regions, Northeastern, Center, and Southern Morocco, were subjected to chemical characterization by LC-ESI/MS^n and NIR spectroscopy and metal determination by AAS spectroscopy. Likewise,

we evaluated the bee venom's bioactive *properties* by assessing its cytotoxic activity on different tumoral cell lines and PLP2 cells and evaluating its anti-inflammatory activity in RAW264.7 cell lines. LC-ESI/MS^n analyses showed that the chemical composition of HBV, mainly the most abundant including melittin, PLA2, and apamin, is notably influenced either by bee environment factors or by collect process conditions. The findings showed no significant differences in mean amounts between or within regions except for PLA2, which is more concentrated in samples from the center and southern region. The data provided by NIR spectroscopy supported this pattern. Heavy metals and micronutrients or trace elements were found in all samples but kept still in the range recommended by guideline references for pharmaceutical raw materials taken into consideration in this investigation (< 5ppm). However, results obtained by AAS spectroscopy revealed that there are, albeit less clear, pollution sources that affected lead concentrations in samples from the northeastern region. We might claim and attribute that to the presence of many coal mines in this region. So, we plan to investigate in this context. NIR and AAS can be highly recommended as two reliable techniques for limit tests and authentication as a pharmaceutical raw. All *Apis mellifera intermissa* venom samples tested on the six tumor cell lines displayed cytotoxic and anti-inflammatory activities. Only samples from the northeastern with the highest concentration mean values in apamin and PLA2 manifested significant activity on MCF-7, MM127, and RAW264.7 cell lines with the lowest concentrations of GI50. Besides, this is consistent to a large extent with the known anti-inflammatory activity essentially for apamin and partially for melittin in addition to synergistic and complementary cytotoxic properties for melittin and PLA2. Furthermore, the correlation test showed that there was a moderate negative linear relationship, on the one hand between PLA2 and MM127 melanoma cell lines and, on the other hand, between PLA2 and MCF-7 tumor cell lines. Likewise, between PLA2

and RAW264.7 and between apamin and the same cell lines. in light of the previous, Apis mellifera intermissa venom can be considered a source of bioactive components. Its evident cytotoxic activity on MM127 and at a moderate level on MCF-7 make it a potential candidate to fight as an adjuvant or treatment, essentially against melanoma skin cancer and to some degree against human breast cancer. This study, being the first report on Apis mellifera intermissa venom from different regions of Morocco, uncovered this valuable product's chemical and biological properties and constituted an attempt to set up a Good Beekeeping Practices (GAP) procedure for bee venom collection. Thus, this confirms its position as the most prone among the enormous bioactive substances for new apiceuticals discovery for melanomas and other tumors treatment.

Keywords: *Apis mellifera intermissa*, honey bee venom, anti-inflammatory activity, cytotoxic activity; melanoma, LC-ESI/MS^n, NIR, AAS

7 Résumé :

Les données ethno-pharmacologiques du Maroc montrent une forte utilisation traditionnelle des tissus et des extraits naturels par nos ancêtres à des fins préventives et curatives, ce qui représente un patrimoine précieux et prometteur pour la recherche scientifique. En effet, ce patrimoine naturel renferme une extrême richesse de la biodiversité en substances bioactives. C'est le cas, par exemple, des produits apicoles tels que le miel, la propolis, la cire et le venin d'abeille. Des manuscrits de l'Égypte ancienne montrent que la momification a été effectuée avec du miel entre autres produits de l'abeille. Dans la culture musulmane, on peut facilement remarquer la part du miel dans l'usage quotidien, en ajoutant bien évidement à tout un chapitre du Saint Coran intitulé "Les abeilles" (Surat An'Nahl). De même, la guérison par les piqûres d'abeilles est bien connue aussi bien dans les cultures orientales ainsi que dans l'Europe ancienne. Dans cette optique, la proposition du présent projet vise à évaluer le venin d'abeille en tant qu'éventuelle substance naturelle bioactive, et à valoriser le produit par la suite, comme adjuvant pharmaceutique et/ou thérapeutique dans le traitement des cancers de la peau particulièrement les mélanomes. Nous prétendons par conséquent que la production de cette substance est un enjeu substantiel et stratégique du développement durable. La caractérisation chimique, la recherche de présence susceptible des métaux lourds et toxiques, ainsi que l'évaluation "in fine" des activités biologiques notamment l'activité anticancéreuse du venin *d'Apis mellifera intermissa*, la principale sous-espèce d'abeille à miel abondante au Maroc constituaient l'axe forgeant de cette étude. Ce travail a suivi une carte de route spécifique comprenant, le développement et l'amélioration d'un dispositif de collecte du venin d'abeille, soumis à une demande de brevet, avec des qualités requises à plusieurs niveaux; couvrant la pureté du venin d'abeille, le rendement de la collecte, la stabilité de la ruche ainsi

que du rucher, la diminution des dommages causés par les abeilles ou la mort des abeilles excitées par période de collecte, en plus, fournir un dispositif abordable pour les apiculteurs marocains. Quinze échantillons de venin d'*Apis mellifera intermissa* provenant de trois régions, le nord-est, le centre et le sud du Maroc, ont été soumis à une caractérisation chimique par CL-IEP/SM^n et par spectroscopie PIR ainsi qu'à la détermination des métaux par spectroscopie SAA. Les propriétés bioactives du venin d'*Apis mellifera intermissa* ont été évaluées en mesurant son activité cytotoxique sur différentes lignées cellulaires tumorales et sur les cellules PLP2, et en évaluant son activité anti-inflammatoire sur les lignées cellulaires RAW264.7. Les analyses CL-IEP/SM^n ont montré que la composition chimique du venin d'abeille, notamment les composants les plus abondants dont la mélittine, le PLA2 et l'apamine, est remarquablement influencée soit par des facteurs liés à l'environnement de l'abeille, soit par des conditions du processus de collecte. Aucune différence significative en quantités moyennes n'a été observée entre les régions ou au sein de celles-ci, à l'exception des quantités en PLA2 qui est plus concentrée dans les échantillons de la région du centre et du sud. Cette tendance est clairement soutenue par les données fournies par la spectroscopie PIR. Les métaux lourds et les micronutriments ont été trouvés dans tous les échantillons mais restent dans les fourchettes recommandées par les références des directives pour les matières premières pharmaceutiques prises en considération dans cette étude (< 5ppm). Cependant, les résultats obtenus par spectroscopie SAA ont révélé qu'il existe des sources de pollution, bien que moins claires, qui ont affecté les concentrations de plomb dans les échantillons de la région nord-est. On pourrait prétendre et attribuer cela à la présence de nombreuses mines de charbon dans cette région. C'est donc dans ce contexte qu'il convient de mener une enquête plus approfondie. La spectroscopie PIR et la spectroscopie SAA peuvent être fortement recommandées comme deux techniques fiables pour les tests limites et pour l'authentification du venin

d'abeille en tant que matière première pharmaceutique. Tous les échantillons de venin d'*Apis mellifera intermissa* testés sur les six lignées cellulaires tumorales ont montré des activités cytotoxiques et anti-inflammatoires, mais seuls les échantillons du nord-est, ayant les valeurs moyennes de concentration les plus élevées en apamine et PLA2, ont montré des activités significatives sur les lignées cellulaires MCF-7, MM127, et RAW264.7 (GI_{50} et EC_{50} plus faible). En outre, cela correspond dans une large mesure à l'activité anti-inflammatoire connue essentiellement pour l'apamine et partiellement pour la mélittine, en plus des propriétés cytotoxiques synergiques et complémentaires pour la mélittine et le PLA2. Par ailleurs, les tests de corrélation ont montré qu'il existe une relation linéaire négative modérée, d'une part entre la PLA2 et les lignées cellulaires de mélanome MM127, et d'autre part entre la PLA2 les lignées cellulaires du cancer du sein MCF-7. Le même constat a été observé entre PLA2 et RAW264.7 et entre l'apamine et les mêmes lignées cellulaires. À la lumière de ce qui précède, le venin d'*Apis mellifera intermissa* peut être considéré comme une mine de composants bioactifs. Son activité cytotoxique évidente sur MM127 et à un niveau modéré sur MCF-7 en font un candidat potentiel pour lutter comme adjuvant et/ou traitement, essentiellement contre le cancer de la peau particulièrement les mélanomes et dans une certaine mesure contre le cancer du sein humain. Cette étude, présentant le premier rapport sur quinze échantillons de venin d'*Apis mellifera intermissa* provenant de trois régions du Maroc, a non seulement mis en évidence les propriétés chimiques et biologiques de ce précieux produit et de le positionner dans l'énorme arsenal de substances bioactives, comme le candidat le plus susceptible à la découverte de nouvelles apiceuticals, mais a également constitué une tentative réussie de mise en place d'une procédure de bonnes pratiques d'apiculture (BPA) en matière de collecte du venin d'abeille, et donc de valoriser cette substance pour devenir une valeur

ajoutée au revenu des apiculteurs et contribuer ainsi au développement durable pour une importante partie de la société.

Mots-clés : *Apis mellifera intermissa*, venin d'abeille mellifère, l'activité anti-inflammatoire, l'activité cytotoxique, mélanomes, CL-IEP/SM^{n}, PIR, SAA

8 Resumo :

Os dados etnoparmacológicos de Marrocos mostram uma forte utilização tradicional de tecidos e extractos naturais pelos nossos antepassados para fins preventivos e curativos, representando um guia valioso e promissor para a investigação. Além disso, o património natural representa uma extrema riqueza de biodiversidade em substâncias bioactivas. É o caso, por exemplo, de produtos apícolas como o mel, própolis, cera de abelha e veneno de abelha. Manuscritos da civilização faraónica mostram que os antigos egípcios mumificaram os seus mortos com mel e outros produtos apícolas. Na cultura muçulmana, podemos facilmente notar a parte do mel em uso quotidiano, e isto está relacionado com um capítulo inteiro do Alcorão Sagrado, sem título "as abelhas" (Surat An'Nahl). A cura por picadas de abelhas é bem conhecida nas culturas orientais, bem como na Europa antiga. Em consonância com isto, a actual proposta de projecto tenta realçar e avaliar o veneno das abelhas como uma possível substância natural bioactiva e, portanto, valorizar o produto como um medicamento em si ou como um adjuvante terapêutico. A partir daí, afirmamos que a sua produção é um desafio substancial e estratégico de desenvolvimento sustentável. O principal objectivo deste trabalho era estudar a caracterização química e avaliar "in fin" o valor potencial das actividades biológicas, essencialmente a actividade anticancerígena do veneno de Apis mellifera intermissa, a principal espécie abundante de abelhas de mel em Marrocos. Este trabalho seguiu um procedimento simples de que a chave estratégica é a melhoria de um colector de veneno de abelha. Apliquemos um pedido de patente para este dispositivo. Esta melhoria afecta vários níveis, incluindo a pureza do veneno das abelhas, o rendimento da recolha, a estabilidade das colmeias no apiário, e a diminuição dos danos das abelhas por período de recolha. Além disso, este colector fornece um dispositivo acessível para os apicultores marroquinos. Quinze amostras de

veneno de Apis mellifera de três regiões, Nordeste, Centro e Sul de Marrocos, foram submetidas a caracterização química por espectroscopia LC-ESI/MSn e NIR e determinação de metais por espectroscopia AAS. Da mesma forma, avaliamos as propriedades bioactivas do veneno da abelha através da avaliação da sua actividade citotóxica em diferentes linhas celulares tumorais e células PLP2 e avaliando a sua actividade anti-inflamatória em linhas celulares RAW264.7. As análises LC-ESI/MSn mostraram que a composição química do HBV, principalmente a mais abundante incluindo a melitina, PLA2, e apamina, é notavelmente influenciada ou por factores ambientais das abelhas ou por condições de processo de recolha. Os resultados não mostraram diferenças significativas nas quantidades médias entre ou dentro das regiões, excepto no caso do PLA2, que está mais concentrado em amostras da região centro e sul. Os dados fornecidos pela espectroscopia NIR suportaram este padrão. Foram encontrados metais pesados e micronutrientes ou oligoelementos em todas as amostras, mas mantidos na faixa recomendada pelas referências de orientação para matérias-primas farmacêuticas tomadas em consideração nesta investigação (< 5ppm). Contudo, os resultados obtidos pela espectroscopia AAS revelaram que existem, embora menos claras, fontes de poluição que afectaram as concentrações de chumbo nas amostras da região nordeste. Podemos afirmar e atribuir isso à presença de muitas minas de carvão nesta região. Por isso, planeamos investigar neste contexto. NIR e AAS podem ser altamente recomendadas como duas técnicas fiáveis para testes limite e autenticação como uma matéria-prima farmacêutica. Todas as amostras de veneno de Apis mellifera intermissa testadas nas seis linhas de células tumorais mostraram actividades citotóxicas e anti-inflamatórias. Apenas amostras provenientes do nordeste com os valores médios de concentração mais elevados em apamin e PLA2 manifestaram actividade significativa nas linhas de células MCF-7, MM127, e RAW264.7 com as concentrações mais baixas GI50. Além disso, isto

é consistente em grande medida com a conhecida actividade anti-inflamatória essencialmente para a apamina e parcialmente para a melitina, além das propriedades citotóxicas sinergéticas e complementares para a melitina e PLA2. Além disso, o teste de correlação mostrou que houve uma relação linear negativa moderada, por um lado entre as linhas celulares de melanoma PLA2 e MM127 e, por outro lado, entre as linhas celulares tumorais PLA2 e MCF-7. Do mesmo modo, entre PLA2 e RAW264.7 e entre apamin e as mesmas linhas celulares. à luz do anterior, o veneno de Apis mellifera intermissa pode ser considerado fonte mineira de componentes bioactivos. A sua evidente actividade citotóxica no MM127 e a um nível moderado no MCF-7 tornam-no um candidato potencial para lutar como adjuvante ou tratamento, essencialmente contra o melanoma cancro da pele e, em certa medida, contra o cancro da mama humano. Este estudo, sendo o primeiro relatório sobre o veneno de Apis mellifera intermissa de diferentes regiões de Marrocos, revelou as propriedades químicas e biológicas deste valioso produto e constituiu uma tentativa de estabelecer um procedimento de Boas Práticas de Apicultura (BPA) para a recolha de veneno de abelha. Assim, isto confirma a sua posição como a mais propensa entre as enormes substâncias bioactivas para a descoberta de novos apiceuticos para o tratamento de melanomas e outros tumores.

Palavras-chave: Apis mellifera intermissa, veneno de abelha, actividade anti-inflamatória, actividade citotóxica; melanoma, LC-ESI/MSn, NIR, AAS

ملخص: 9

تظهر معطيات الإثنوغرافية الدوائية بالمغرب استخداما تقليديا للأنسجة والمستخلصات الطبيعية من قبل أسلافنا لأغراض وقائية وعلاجية الأمر الذي يجعله مرشدا واعدا للبحث العلمي، إلى جانب الإرث الطبيعي الذي يحوي ثروة هائلة من التنوع البيولوجي في المواد الفعالة بيولوجيا و خير مثال على ذلك منتجات النحل كالعسل، و العكبر، و الشمع، وسم النحل .

المخطوطات المصرية القديمة تثبت أن التحنيط كان يتم، إلى جانب مواد أخرى، بالاستعانة أساسا بمادة العسل ومنتجات الخلية الأخرى. ويمكن للمرء أن يلاحظ بسهولة الاستخدام اليومي للعسل في الثقافة الإسلامية خاصة لارتباطه بسورة كاملة من القرآن الكريم و يتعلق الأمر بسورة النحل، بالإضافة الى أن التداوي بلسعات النحل كان معروفا بشكل جلي في الثقافات الشرقية وكذلك في أوربا القديمة.

اعتبارا لكل ما سبق، يندرج هذا المشروع، كأول بحث علمي أكاديمي متكامل، في سياق تثمين مادة سم النحل كأحد المكونات الطبيعية و الفعالة بيولوجيا واستخدامه لاحقال كمكمل علاجي أو مصدر لإنتاج مستخلصات دوائية فعالة قائمة بذاتها في علاج الأورام الجلدية خاصة الورم القتاميني (الميلانوما)، و لذلك نطمح أن يكون تثمين هذه المادة في إطار هذا المشروع العلمي، قاطرة لاستراتجية مدروسة لتطوير سلسلة تربية النحل من أجل تنمية مستدامة .

تتمحور هذه الدراسة حول التوصيف الكيميائي والبحث عن وجود محتمل للمعادن الثقيلة والسامة إضافة إلى التقييم الدقيق للنشاط البيولوجي لسم نحلة العسل الأنترميسا، والتي تشكل سلالة النحل الرئيسية في المغرب، لاسيما النشاط المضاد للأورام.

اتبع هذا العمل خارطة طريق محددة تتمثل في تطوير وتحسين جهاز جمع سم النحل الذي يخضع حاليا لطلب براءة الاختراع ،هذا الجهازيتميز بخصائص جودةعلى عدة مستويات، تشمل أساسا نقاء سم النحل المحصل عليه اثناء عملية الجمع ،استقرار الخلية، انخفاض الأضرار الناجمة عن موت النحل اثناء فترة الجمع، و كذلك توفير جهاز مصنوع محليا بأسعار معقولة لمربي النحل.

خمسة عشر عينة من سم نحلة العسل الأنترميسا أخدت من ثلاثة مناطق مختلفة بالمغرب؛ الشمال الشرقي، الوسط ،والجنوب. تم إخضاع هذه العينات للتوصيف الكيميائي بطريقة مزاوجة التحليل اللوني السائل و التحليل الطيفي الكتلي ($LC\text{-}ESI/MS^n$) و تحليل المطيافية تحت الحمراء القريبة (NIR spectroscopy) إضافة إلى تحديد و قياس المعدن خاصة الثقيلة و السامة بواسطة تحليل مطيافة الامتصاص الذري (AAS).

إضافة إلى ذلك، تم تقييم الخصائص الفعالة بيولوجيا لسم نحلة العسل الأنترميسا عن طريق قياس القدرات السمية الخلوية على عدة سلالات من الخلايا السرطانية المختلفة وعلى سلالات خلايا طبيعية مستخلصة من كبد الخنزير الداجن(PLP2) وعن طريق تقييم النشاط المضاد للالتهابات على سلالات خلايا RAW264.7 .

أظهرت الدراسة ان تركيبة سم النحل خاصة المكونات الأساسية منها بما في ذلك الأبمين، و الفوسفوليبا-A2 تتأثر بشكل واضح إما بالعوامل المتعلقة ببيئة النحل أو بظروف عملية الجمع. تحليل المعطيات و نتائج التحاليل المخبرية بين أنه لم يلاحظ وجود فارق واضح في القيم المتوسطة لهذه المواد في العينات المأخودة من مختلف المناطق أو داخل نفس

المنطقة باستثناء مادة الفوسفوليباز-A2 التي كانت أكثر تركيزا في عينات الجهة الوسطى و الجنوبية ، هذه النتائج تدعمها البيانات المحصل عليها من خلال تحليل المطيافية تحت الحمراء القريبة (NIR spectroscopy).

تم العثور على المعادن الثقيلة و المغذيات الدقيقة أو العناصر النادرة في العينات لكنها تظل ضمن النطاقات التي أوصت بها مراجع المبادئ التوجيهية للمواد الخام الصيدلانية التي تم اعتمادها في هذه الدراسة (اقل من ppm5) لكن النتائج التي تم الحصول عليها عن طريق التحليل المطيافي للامتصاص الذري (SAA) أظهرت أن هناك مصادر للتلوث وإن كانت واضحة مما جعل تركيز الرصاص في عينات المنطقة الشرقية أكبر بالقارنة مع باقي عينات المناطق الأخرى. ويمكن ان يعزى ذلك الى وجود العديد من مناجم الفحم في هذه المنطقة لهذ قررنا في المستقبل القريب بإجراء بحث ميداني في هذا الشأن. على ضوء ما سبق يمكن اعتبار تقنية المطيافية للأشعة التحت حمراء القيبة (NAR Spectroscopy) طريقة فعالة و إيكواوجية لاختبار الجودة لسم النحل كمادة أولية صيدلانية و كذا تتبع مصدرها و مدى طراوتها.

أظهرت جميع عينات سم نحلة العسل الأنترميسا التي تم اختبارها على سلالات الخلايا السرطانية الستة نشاطا سميا خلويا و مضادا للالتهابات. لكن فقط عينات الجهة الشمالية الشرقية هي التي أظهرت نشاطا واضحا على سلالات الخلايا السرطانية MCF-7 MMI127 وكذاسلالة خلايا RAW264,7 (قيم EC_{50} و GI_{50} الأضعف). يتوافق هذا إلى حد كبير مع النشاط المضاد للالتهابات المعروف بشكل أساسي لدى الأبتامين وثانوي بالنسبة الملتينين. بالإضافة إلى الخصائص التآزرية و المضاضة لتطور الخلايا السرطانية للملتينين والفوسفوليباز A2.

علاوة على ذلك أظهرت اختبارات الارتباط وجود علاقة خطية معتدلة سلبية من جهة، بين الفوسفوليباز A2 وسلالات الخلايا السرطانية الجلدية الميلانوما MMI127 ومن جهة اخر بين الفوسفوليباز A2 وسلالات خلايا سرطان الثدي MCF-7 وبين الفوسفوليباز A2 . نفس الملاحظة بالنسبة للفوسفوليباز A2 و سلالة خلايا RAW264,7 وأيضا بين الأبامين ونفس سلالات الخلايا قيد الدراسة.

خلاصة يمكن اعتبار سم نحلة العسل الأنترميسا بمثابة منجم للمكونات الفعالة بيولوجيا اعتبارا لنشاطه السمي الخلوي الواضح على سلالات الخلايا MMI127 ونشاطه المعتدل على سلالات الخلايا MCF-7 مما ترشيحه للإستعمال الدوائي كعلاج موازي أو كعلاج أساسي ضد سرطان الجلد الميلانوما أساسا، أوإلى حد ما ضد سرطان الثدي عند الإنسان.

هذه الدراسة التي قدمت تقريرا أوليا عن خمسة عشرة عينة من سم نحلة العسل الأنترميسا من ثلاث مناطق مختلفة بالمغرب لم توضح فقط الخصائص الكيميائية والبيولوجية لهذا المنتج الثمين و وضعه ضمن ترسانة هائلة من المواد الفعالة بيولوجيا وجعله المرشح الأكثر احتمالا لإنتاج مستحضر علاجي جديد من أصل منتوجات خلية النحل، بل كذلك محاولة ناجحة لتأسيس إجراءات الممارسات الجيدة لتربية النحل لعملية جمع السم و بالتالي تثمين هذه المادة لتصبح قيمة مضافة لدخل النحالين و بالتالي المساهمة في التنمية المستدامة لمجموعة واسعة من المجتمع.

الكلمات الأساسية: نحلة العسل الأنترميسا، سم نحلة العسل، النشاط المضاد للالتهاب، النشاط السمي الخلوي، الورم القتاميني (الميلاني)، التحليل المطيافي بالأشعة تحت الحمراء القريبة، التحليل المطيافي للامتصاص الذرية، التحليل اللوني السائل و التحليل الطيفي الكتلي.

الكلمات المفتاحية: أبيس ميليفيرا أنتيرميسا، سم نحل العسل، النشاط المضاد للإلتهاب، القدرات السمية ابخلوية، سرطان الجلد، التحليل المطيافي للإمتصاص الذري، التحليل المطيافي للأشعة تحت الحمراء القريبة، التحليل اللوني السائل\التحليل الطيفي الكتلي.

Chapter I: Introduction.

10 Honey bee Apis mellifera species:

10.1 Taxonomy and geographical distribution over the Morocco territory:

The honey bee *Apis mellifera* species are a member of the kingdom of Animalia, the phylum Arthropoda, the class Insecta, the order Hymenoptera (from the Greek hymen, for membrane, and pteron, for wing), the family Apidae, the subfamily Apinae, the genus Apis (1). Thirty-three distinct honey bee subspecies are distributed across all of Africa (11 subspecies), western Asia and the Middle East (9 subspecies), and Europe (13 subspecies). All honey bee subspecies are subdivided into five evolutionary lineages, supported by morphometric and genetic studies in addition to analysis of ecological, physiological, and behavior traits: lineage A (10 subspecies) and its sublineage Z (3 subspecies) lineage M (3 subspecies), lineage C (10 subspecies), lineage O (3 subspecies), lineage Y (1 species), and lineage C or O (2).

The Morocco honey apifauna comprised three different races, including *Apis mellifera intermissa* "the Tellian," one of the "big" mellifera races, located northwest of the Atlas range. *Apis mellifera major* found in the areas close to the Mediterranean coast and the Rif mountains of Northwest Morocco. This bee may be a brown variety of the *Apis mellifera intermissa,* but there are also anatomic differences. *Apis mellifera Sahariensis* at the southeast of the Atlas range and in the north oasis near the northern edge of Morocco Sahara. The geographical distribution of the three races is depicted in Figure 1 (1–4). Transition zones between the areas of subspecies have been shown, which led to gradient changes in morphometric and molecular features. On the one hand, between *Apis mellifera intermissa* and *Apis mellifera major*, and on the other

hand, between *Apis mellifera intermissa* and *Apis mellifera major* (2). Hence we can encounter local hybrids or ecotypes more adapted to their biotopes (1,3).

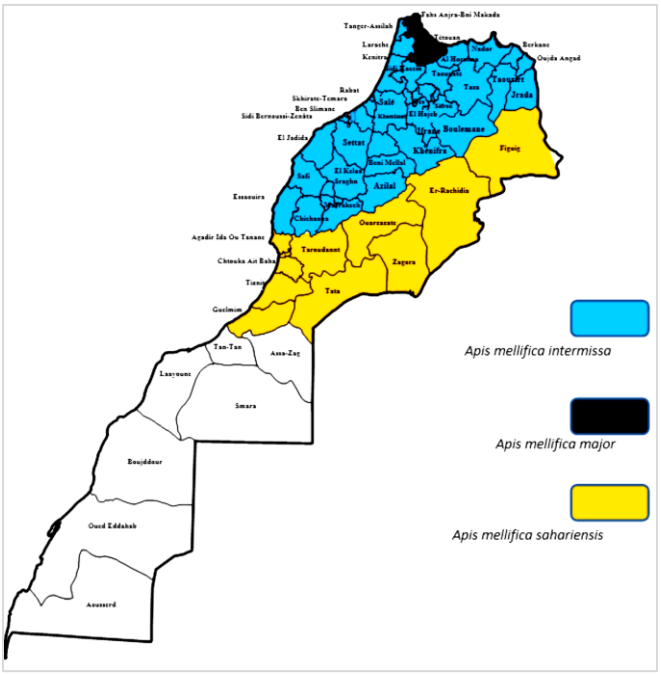


Figure 1. Geographical distribution of the three *Apis mellifera* endemic races in Morocco

10.2 Characteristics:

Apis mellifera intermissa (Figures 2, and 3), the subspecies subjected to the present study, phonetically takes the linking position between races in the north (*Apis mellifera sicula* and *Apis mellifera Iberica*) and those from the south (*Apis mellifera sahariensis*) (4). This bee is overly aggressive, and nervous and produces many broods with many queen cells. *Apis mellifera intermissa* is characterized by a long body, dark pigment, sparse hairs, tongue length of 6.4 mm, and prone to swarming (1). additional feature details are depicted in Table 1.

These characteristics can be variate by environmental and feeding conditions, such as the impact of artificial feeding, which acts as a stress factor affecting wing venation symmetry of worker honey bees (5).

Figure 2. *Apis mellifera intermissa* (https://en.wikipedia.org/wiki/Apis_mellifera_intermissa)

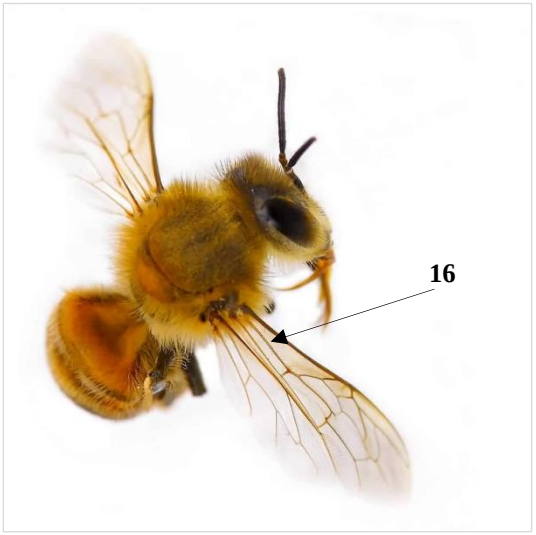


Figure 3. Angel 16 of right forewing venation of a worker honey bee *Apis mellifera* (https://www.perfectbee.com/)

Table 1. Characteristics of *Apis mellifera intermissa* (Tellian bee) (1).

Characters	***Apis mellifera intermissa***
Proboscis (mm)	6.38
Hind leg (mm)	8.12
Length of the forewing (mm)	9.18
Length of tergite 3 + 4 (mm)	4.43
Color of tergite 4 (Scale from 0.0 = completely dark to 9.0 = completely yellow)	1.1
Cubital index	2.22
Angel of wing venation 16 (Fig.3)	95.76

10.3 Sting apparatus:

The social castes are well determined in a bee colony. The critical element in this complementary unit is, of course, the worker bee. This latter is organized by many subcastes which each one engaged in a specific task. The house bee castes sweat wax and become guard bees that guard the hive with their stingers by injecting bee venom into the skin or an intruder's body. Only queen and worker bees detain sting apparatus (Figure 4) and make venom in their venom gland, which is then stored in the venom sac located next to the stinger. These structures (Figure 5) are located in the abdomen's posterior region between the rectum and ovaries. The venom gland is an extended tubular filamentous region (acid gland) connected to a reservoir at its proximal region in which the venom is stored. The distal end of the reservoir or venom sac is connected to the sting bulb, which leads into the venom canal present inside the sting's shaft (1,5,6). Table 2 describes different morphometric parameters of the sting apparatus of the honey bee *Apis mellifera*. Differences and similarities can be shown within the same subspecies (5).

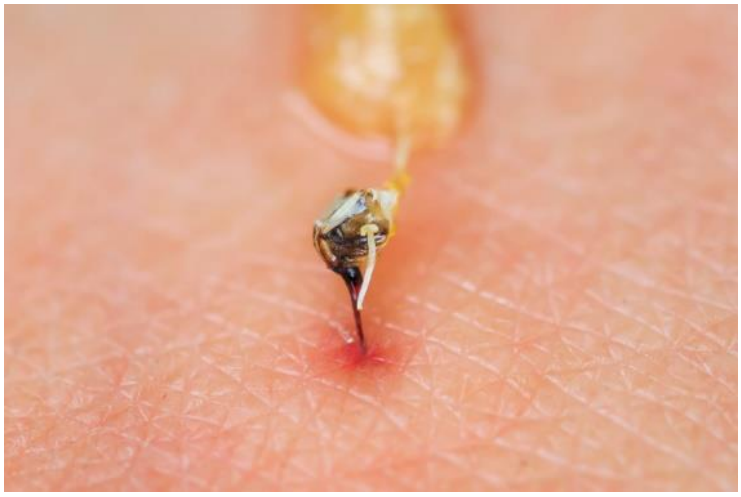


Figure 4. Honey bee sting Apparatus (https://www.shutterstock.com/image-photo/)

Table 2. Different morphometric parameters of the sting apparatus of honey bee *Apis mellifera*

Morphometric Parameters	Apis mellifera species mean ± SD (mm)
Sting length	4.20 ± 0.03
Sting width	2.92 ± 0.02
Quadrate plate length	1.06 ± 0.00
Quadrate plate width	0.90 ± 0.00
Oblongate plate length	1.40 ± 0.00
Oblongate plate width	0.5 ± 0.00
Triangular plate length	0.41 ± 0.00
Triangular plate width	0.26 ± 0.00
Stylet length	2.10 ± 0.02
Stylet width	0.27 ± 0.00
Lancet length	0.12 ± 0.00
Lancet width	0.16 ± 0.02
Venom gland length	17.58 ± 0.22
Venom gland width	0.07 ± 0.01
Venom sac length	4.42 ± 0.10
Venom sac width	1.48 ± 0.30
Dufour's gland	0.92 ± 0.10
Dufour's gland with	0.30 ± 0.01

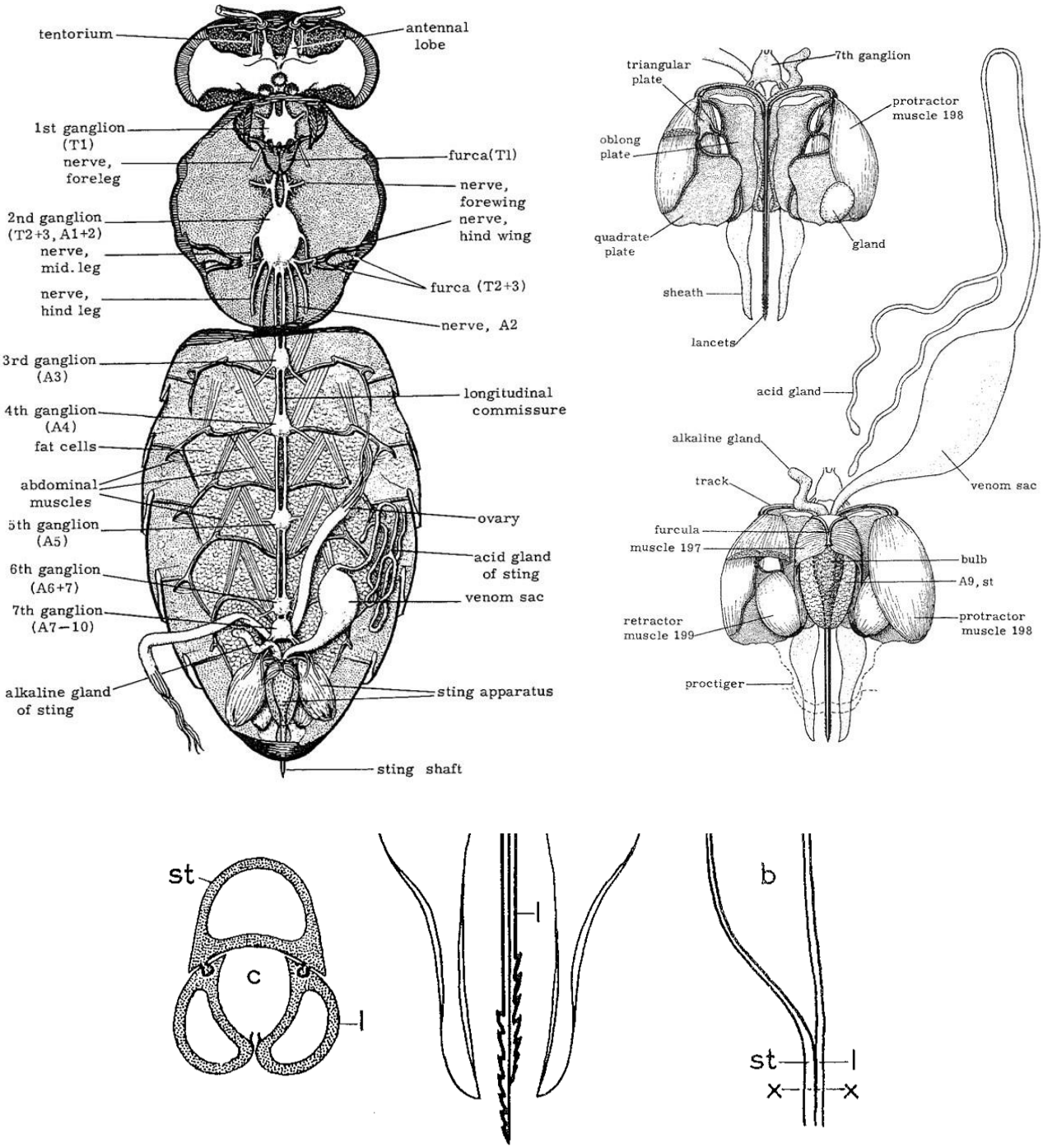


Figure 5. Anatomy and dissection of the sting apparatus of honey bee *Apis mellifera*. On the right, dissection of a worker, Stage 3: head canted back to show some parts more clearly, glands removed; alimentary canal removed to expose the nervous system, sting apparatus, and floor of the abdomen. On the left, the sting apparatus: dorsal aspect, as in Plate 10. Above: ventral aspect, revealed when apparatus is lifted out and turned over. Below: on the right, a longitudinal section through part of the bulb and shaft; on the left, a cross-section through the shaft. v, duct of venom gland; b, bulb; l, lancet; o, oblong plate; q, quadrate plate; t, triangular plate; 1r, first ramus; 2r, second ramus; st, stylet; c, venom canal (6).

11 Honey bee venom :

11.1 Chemical and biological activities of Honey bee venom:

11.1.1 HBV chemical composition and biological properties:

Honey bee venom (HBV) is a biotoxin or apitoxin synthesized and secreted by the venom gland located in the honey bee's abdominal cavity (7). It has been suggested that HBV is well beyond the classical stereotype of defense against predators (8). It also acts as a medium of social antisepsis, contributing to collective immunity, and it is prominent for the colony. Even without complete scientific proof of efficacy and safety, it has been used in traditional medicine (in certain countries) to prevent or treat many diseases such as arthritis, rheumatism, pain cancer, skin problems, and periodontal diseases. Moreover, it acts as an anti-inflammatory, leishmanicidal agent, antimicrobial, anti-viral, antiapoptotic, wound healer, antifibrinolytic, and anti-elastolytic (9–16). Furthermore, HBV can improve the reproductive performance (natural sexual stimulant) and immune response (natural immunostimulant) of mal rabbits, particularly under high-temperature conditions (17). The HBV components have a wide range of pharmacological targets and have been extensively studied as promising sources of new drugs and biopesticides (18). Table 3 describes the main biological activities and therapeutic effects of HBV and its components. HBV is an intricate liquid mixture of biologically active substances comprising proteins, peptides, enzymes, and other small molecules (12,14,19,20). 0.1μg of dry venom is extractable from a single bee (13,21). This product has more than 80% water, about 3% of volatile substances, and 12% of its dry weight (Fig.6).

A more in-depth description will be given for the most relevant compounds present in HBV, considering the number of studies in the literature and different techniques for its evaluation.

Table 3. The main biological activities and therapeutic effects of HBV

Components	Biological activity and therapeutic interests		References
Melittin	anticancer activity	breast cancer MCF-7, T47D, MDA-MB-23, SKBR3, SUM159, and SUM149, cell lines	(66–74)
		human gastric cancer SGS-7901	(75)
		hepatocarcinoma HpG2 cell lines, human cervix carcinoma HEP-2C cell lines,	(66,76,77)
		Acute lymphoblastic leukemia (CCRF-CEM) and chronic myelogenous leukemia (K-562)	(78)
		human cervical carcinoma CaSki, HeLa cell lines, C33A, and TC-1cell lines	(79–82)
		human ovarian cancer A2780 and A2780CR, SKOV3, and AP-1 cell lines	(83–89)
		U118 glioblastoma multiform cell lines	(90)
		human renal cancer caki-1 cells, human bladder cancer TSGH-8301cells, human prostate cancer LNCaP, DU145, and PC-3 cell lines	(72,91,92)
	anti-inflammatory effects		(93–100)
	anti-microbial activity		(101–104)
Apamin	anti-inflammatory effects		(105)
MCDP	lyses mast cells, releases histamine, serotonin, and heparin, increases capillary permeability, anti-inflammatory, analgesic effect, simulates the central nervous system		(29,67,106–108)

Secapin	antibacterial, anti-fibrinolytic, and anti-elastolytic, inflammatory effects and pain induction through the lipoxygenase pathway	(109–111)
Adolapin	inhibition of PLA2 and COX activity, anti-inflammatory activity, inhibits the specific brain enzymes cyclooxygenase and lipoxygenase, decrease of inflammations by, anti-rheumatic, decrease of pain, inhibits the aggregation of erythrocytes, antipyretic	(8,67,107,108)
Tertiapin	inhibitor of kir1.1 and kir3.1/3.4 channels	(35)
Procamine	antiarrhythmic effects	(106)
Minimine	undefined	
PLA2	anti-microbial, immunomodulatory, anti-inflammatory, destroys phospholipids and dissolves the cell membrane of blood bodies, decreases the blood coagulation and blood pressure, prevents neuronal cell death caused by prion peptides, cytotoxic effects against cancer cells, antitumor effects	(67,106,107,112)
Hyaluronidase	catalyzes the hydrolysis of proteins, dilates blood vessels, and increases permeability, causing an increase in blood circulation, immune response, tissue spread activity	(8,67,107,108)

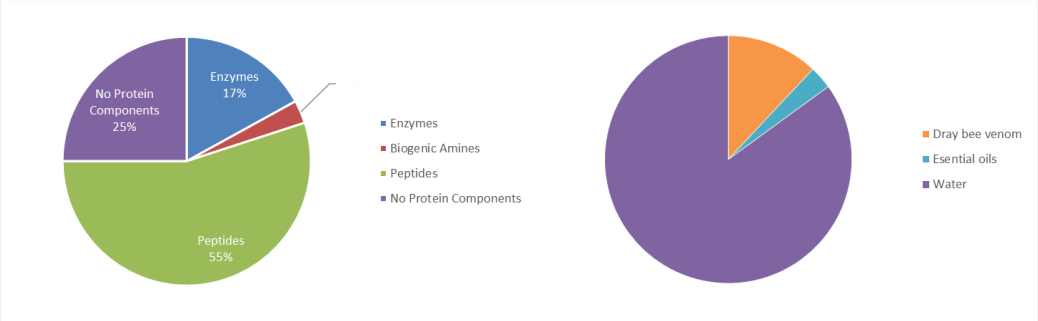


Figure 6: Chemical composition of dried honey bee venom (left) and fresh extract (right)%.

11.1.1.1 Melittin:

Melittin (Fig.7) is the main component of HBV and one of the most studied archetypes of membrane proteins. It consists of 26 amino acid residues. Melittin is a cationic peptide with six positive charges. Four of these charges are in the highly basic C-terminal tetrapeptide sequence Lys^{21}-Arg^{22}-Lys^{23}-Arg^{24} ($K_{21}RKR_{24}$) with only the N-terminal amino group and Lys-7 in the N-terminal sequence. The amphiphilic property of this peptide makes it water-soluble and spontaneously associable with natural and artificial membranes. Consequently, it becomes a famous agent for studying peptide-lipid interaction (22–24). Melittin causes selective micellization of the lipid bilayer, and membrane fusion at high concentrations (10,25,26) as well as it is believed to be the cause of hemolytic activities. This peptide also shows antimicrobial activity in interaction with DMPC (1,2-Dimyristoyl-sn-glycero-3-phosphocholine) membranes and an anti-inflammatory effect on muscle injuries (27,28).

11.1.1.1 Apamin:

Apamin is the smallest peptide and the major neurotoxin in HBV (29). It is an octadecapeptide that contains four cysteine residues with two disulfide bonds

(Cys[1]-Cys[11]) and (Cys[3]-Cys[15]) (26), resulting in the stabilization of its tertiary structure for the expression of biological activity (30).

Figure 7. NMR chain A melittin structure according to PDBe, RCSB PDB, PDBj, and PDBsum (https://www.rcsb.org/3d-view/2MW6)

The presence of the disulfide bridges and the positive charges of arginine residues at positions 13 and 14 of the peptide is essential to its biological activity (Figure 8). Regardless of the route of administration (31), Apamin is the only known polypeptide that crosses the blood-brain barrier (32) and produces motor hyperexcitability (12). Apamin is a specific central neurotoxic, whose activity is due to its ability to block small conductance Ca^{2+}-activated K^{+} channels. It also exerted an anti-inflammatory effect on gouty arthritis in the mouse model (33,34).

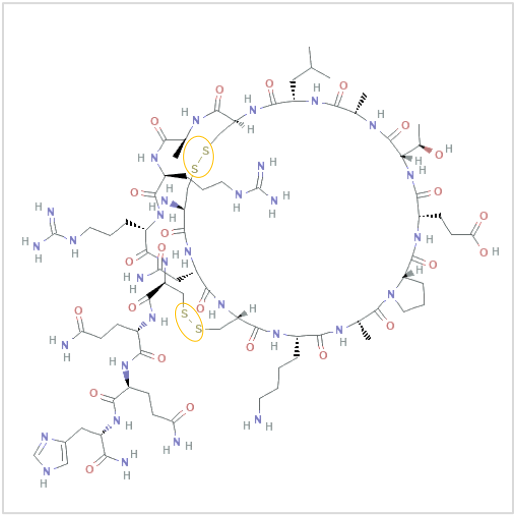


Figure 8. 2D chemical structure of *Apis mellifera* apamin (https://pubchem.ncbi.nlm.nih.gov/compound/16133797)

11.1.1.2 Mast Cell Degranulating Peptide (MCDP) :

MCDP is a 22 residues polypeptide, stable over pH 2-8, with two disulfide bridges and an amidated C-terminal carboxyl group. MCDP is an extremely basic and exceedingly hydrophilic polypeptide possessing a net charge of (8+) (35). MCDP has powerful *in vitro* degranulating effects on mast cell granules by merging the granule membranes with the mast cell membrane and the exocytosis of the granule contents without lysis of mast cells (12,31,36,37). MCDP has a potent anti-inflammatory activity (at doses as low as 0.1 mg/kg) in various animal models. It is not a real anti-inflammatory agent but exerts its action via the mediation of mast cell-derived species (37).

11.1.1.3 Secapin:

This a polycationic peptide with 25 amino acid residues long isolated from different *Apis mellifera* subspecies. It is stabilized by an intramolecular disulfide bridge formed between Cys^{9} and Cys^{20} residues (38–40). Secapin has been shown in two isoforms, secapin-1 and secapin-2, with a similar secondary structure. *Apis cerana* secapin-1 exhibited anti-microbial activity against fungi and gram-positive and gram-negative bacteria. Moreover, it has shown a fibrinolytic activity and acts as an anti-elastolytic factor (41). Whereas, Secapin-2 can induce hyperalgesia and edematogenic responses (42).

11.1.1.4 Adolapin:

Adolapin is a basic polypeptide with 103 amino acid residues, exhibiting an anti-nociceptive and anti-inflammatory activity by inhibiting cyclo-oxygenase function. Furthermore, it has shown an antipyretic and a potent analgesic effect. These latter are presumably due to their capacity to inhibit the prostaglandin synthetase system, following a biphasic dose-response relationship (43–45).

11.1.1.5 Tertiapin:

Tertiapin is a presynaptic neurotoxin peptide composed of 21 amino acid residues. A trace peptide containing two disulfide bridges and a C-terminal residue in an amidated form (46). The single methionine residue in tertiapin (M13) can be readily oxidized. Consequently, its affinity for inhibiting specific inward-rectifier K^+ channels will be 4 to 5-fold lower (47,48). Furthermore, tertiapin presented high-affinity binding sites in the rat brain. However, it can inhibit the enzyme-activating capacity of calmodulin and the activity of soluble phosphodiesterase (45,49,50).

11.1.1.6 Procamine and minimine:

Procamine, with only five residues, has been isolated for the first time from Canadian honey bees as histamine-terminal peptides (51). Procamine-A AGPQ-histamine and procamine-B AGQG-histamine might be related to the radioprotective properties attributed to bee venom (52). Minimine is a basic peptide with 48 to 52 amino acid residues. So far, this peptide's chemical properties and biological activities remain unknown (13,53).

11.1.1.7 Phospholipase A2 (PLA2):

PLA2 (Figure 9), the primary allergen compound of bee venom, is a glycoprotein and consists of a single chain of 128 amino acid residues containing attached carbohydrate residues (54). A revised study based on the analysis of the cDNA for PLA2 from the HBV gland showed that the sequence of the mature bee venom PLA2 comprises 134 amino acids residue with a single glycosylation site at Asn13, which is cross-linked by four disulfide bridges responsible for their stability and also for their folding mechanism (55–57). Bee venom PLA2 belongs to the secreted group III sPLA2 enzymes. It shows its inflammatory activity by inducing the biosynthesis of prostaglandin, arachidonic, and lysophosphatidic

acids by catalyzing the specific hydrolysis of the ester bond at the C2 position 1,2-diacyl-3-sn-glycerophospholipids (55–63).

11.1.1.8 Hyaluronidase:

Properly called hyaluronoglucosaminidase (Hya) (52). It has no direct toxicity (64) but plays a role as a spreading factor since the depolymerization and the hydrolysis of the substrate facilitate the diffusion of other venom constituents (52,65,66).

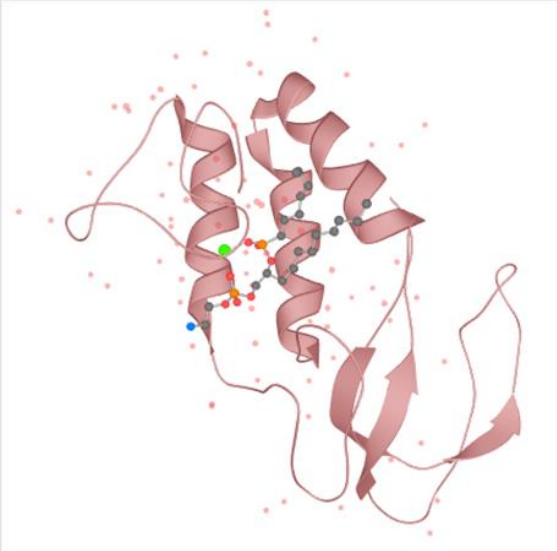


Figure 9. X-ray chain A structure of *Apis mellifera* PLA2 according to PDBe, RCSB PDB, PDBj, and PDBsum

Bee venom Hya is a glycoprotein composed of 349 amino acids containing four cysteines with two disulfide bridges: Cys^{189}-Cys^{201} and Cys^{22}-Cys^{313} (65,67).

Bee venom Hya shares a 30% sequence identity with human hyaluronidase, which is involved in fertilization and hyaluronan turnover (68).

11.1.1.9 Volatile and pheromone components:

Unfortunately, there is an insufficiency of knowledge about volatile compounds in HBV. Depending on the bee species, qualitative and quantitative differences in these substances have been reported (69). Likewise, differences in the effectiveness of individual compounds in eliciting alarm responses in the same species have been demonstrated (70). Over 40 volatile and pheromonal blende

components have been unveiled. (z)-11-eicosen-1-ol, iso-pentyl acetate, n-octyl acetate, benzyl acetate, iso-pentanol, 2-nonalol, n-hexyl acetate, benzyl alcohol, n-butyl acetate, and n-decyl acetate are the major of in single honey bee (70,71). Isoamyl acetate (1μg/sting), one of the main components of the adult bee sting volatiles, is absent in queens and newly hatched bee workers (72). Besides, (z)-11-eicosen-1-ol, a primary volatile compound, shows capabilities to prolong the effectiveness of isopentyl acetate. (73).

11.1.1.10 Metals:

The composition of bee venom may contain several metal elements. Kokot et al. (74) reported the occurrence of microelements such as Cu, Zn, B, Al, V, Mn, Co, Ni, Sr, and Mo, macro elements such as K, Na, Ca, and Mg as well as toxic metals which included As, Ba, Cd, Sb, Cr, and Pb. They suggested that these metals' content depends on species, time, and season but observed that the amounts do not surpass the premised levels established on pharmacopeias (74). The identification of metal contamination, mainly the most toxic, is highly recommended by the authors to guaranty the safety, conformity, and quality of bee venom as a pharmaceutical raw material. Unfortunately, there is a noteworthy insufficiency of data concerning metal contamination in the HBV literature (75). According to the European Directorate for the Quality of Medicines (EDQM) (Table 4), lead, mercury, ruthenium, palladium, antimony, copper, silver, cadmium, bismuth, gold, platinum, vanadium, arsenic, tin, and molybdenum must be identified in all substances for pharmaceutical use (76).

Table 4. Heavy metals subjected to limit tests in pharmaceutical use substances according to the EDQM

Lead (Pb)	Mercury (Hg)	Ruthenium (Ru)	Palladium (Pd)	Antimony (Sb)
Copper (Cu)	Silver (Ag)	Cadmium (Cd)	Bismuth (Bi)	Gold (Au)
Platinum (Pt)	Vanadium (V)	Arsenic (As)	Tin (Sn)	Molybdenum (Mo)

11.1.1 HBV biological activities:

11.1.1.1 Cytotoxic and anticancer activities:

Among animal venoms, honey bee venom has been demonstrated to be the most studied regarding its cytotoxicity and anticancer activities (77). Studies have linked HBV to various cancer management effects, including induction of apoptosis, necrosis, cytotoxicity, and cell proliferation inhibition in many cancer types. These findings were obtained either by evaluating the whole honey bee venom or its main polypeptides on animal cancerous cell lines or human tumor cell lines.

Likewise, these studies were performed *in vitro* or *in vivo* on animal models. It has been demonstrated that HBV and their major proteinic component Mlt and PLA2 acquire cytotoxic and anticancer activities by inhibiting or inducing directly or indirectly many signaling pathways (7,78–82). Overall, HBV, Mlt, and PLA2 can affect the six hallmarks of cancers, including sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (83).

11.1.1.1.1 Breast Cancer:

According to the World Health Organization, breast cancer is the most frequent cancer in women, impacting 2.1 million women each year, and also causes the

most significant number of cancer-related deaths among women (84). Breast cancers represent a critical public problem that requires further research at the molecular level to define its prognosis and specific treatments. On the one hand, MCF-7 (luminal breast cancer cells), a primary tumor, non-invasive breast ductal carcinoma which represents a model of early-stage disease due to the presence of functional estrogen receptors (ER) and estrogen dependence for growth both *in vitro* and *in vivo* (85,86). On the other hand, MDA-MB-231 (HER2-enriched breast cancer) is an overly aggressive, invasive, and poorly differentiated triple-negative breast cancer cell line as it lacks estrogen ER and progesterone receptor (PR) expression, as well as HER2 (Human epidermal growth factor receptor 2) amplification (85). These two breast cancer cell lines proved to be suitable model cell lines for breast cancer investigations worldwide, including those regarding anticancer drugs (86). It has been reported that HBV and Mlt showed a significantly high cytotoxic activity against the MCF-7 cell line with 50% inhibition concentration (IC_{50}) of 2.85 μg/mL and 1.12 μg/mL, respectively (87). Moreover, HBV and Mlt increase the expression of p53 and decrease that of Bcl-2 genes. It has been found that only HBV increases the level of Bax gene expression. This finding may be due to the presence of PLA2 in crude bee venom, given that this latter exhibits its cytotoxic effect through its activation by Mlt (79). Concordant results have been demonstrated in addition to the fact that HBV showed a significant decrease in mRNA level of the two breast cancer-related receptors EGFR and ERα (88). Furthermore, HBV arrested MCF-7 and T47D (luminal breast cancer), a human hormone-dependent breast cancer cell line, originally derived from the same metastatic site of pleural effusion that the MCF-7 cell lines, in the G2/M phase of the cell cycle (89).

Investigations unveiled at a molecular level the HBV and Mlt inhibitory effects on invasion and metastasis of MCF-7, MDA-MB-23, and SK-BR-3 cells, as depicted in Figure 10 (90,91). The biological processes of metastasis rewire

extracellular matrix (ECM) destruction, including mesenchymal collagen and endothelial basement membrane. The prominent ECM degradation enzyme families are matrix metalloproteinases (MMPs). Among the MMPs, MMP-9 is known to be involved in the degradation of type IV collagen, an essential ECM cell motility, and metastasis component.

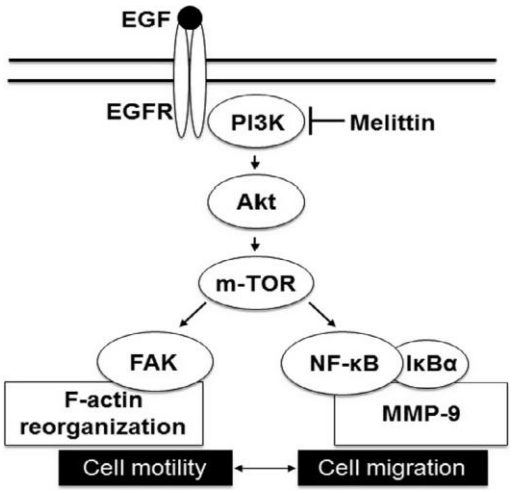


Figure 100. Schematic model for suppression of cell motility and invasion by Mlt in the breast cancer cells.

Findings showed that HBV and Mlt have an inhibitory effect on cell motility by preventing F-actin reorganization in the studied cell lines, which is associated with the inhibition of epithelial-to-mesenchymal transition in breast cancer cells. HBV and Mlt inhibited, in a dose-dependent manner, the EGF-induced activation of MMP-9 and FAK phosphorylation, one of the critical genes for motility and metastasis, which is found to be overexpressed in breast cancer (92,93). But it has seemed that the antineoplastic-key activity is contributed to the Mlt peptide. Likewise, Mlt inhibited the ERK phosphorylation among the Mitogen-activated protein kinas (MAPKs) and decreased the EGF-induced JNK and *p*38 MAPK phosphorylation in the MCF-7 cells. Mlt significantly suppressed EGF-induced PI3k/Akt phosphorylation in the MDA-MB-231 and

MCF-7. Moreover, Mlt drastically suppressed the EGF-induced mTOR phosphorylation in both cancerous cell lines and inhibited the phosphorylation of p70S6K and 4E-BP1, which are the best-characterized effector molecules and downstream molecules of mTOR. Mlt inhibited the p65 (a subunit of NF-κB) translocation in a dose-dependent manner, and thus, NF-κB downregulated (92,93).

By RMAN-monitoring the biochemical changes in MDA-BM-231 breast cancer cells following HBV treatment, it has been demonstrated that HBV reduced in a time- and dose-dependent manner the expression level of caspase-8, caspase-9, and caspase-3 as well as the poly-(ADP-ribose)-polymerase (PARP). Remarkable morphological changes were observed from 3 μg/mL as well (94).

In their work, Duffy et al., 2020, pinpointed the underpinning anticancer molecular mechanism of HBV and Mlt in the aggressive triple-negative and HER2-enriched breast cancer subtypes. In this study, the authors demonstrated that HBV and Mlt remarkably reduced the cell viability with high anticancer selectivity, with significantly higher potency in TNBC (SUM159 and SUM149) and the HER2-enriched breast cancer cell lines (MDA-MB-453 and SKBR3), followed by luminal breast cancer (MCF7 and T-47D) with negligible impact on normal cells (primary dermal fibroblast cells HDFa, mammary nontransformed MCF 10A, and MCF-12A cells). Moreover, in comparison to normal cells, cell viability assays of HBV and Mlt in murine breast cancer and normal cell lines confirmed enhanced selectivity for aggressive murine tumor cell lines, such as the p53-mutant claudin-low T11 and BRC A-mutant B15. According to the bioluminescence resonance energy transfer assay (BRET), Mlt reached the kinases by pore-formation into the plasma membrane of cancer cells via the charged sequence ($K_{21}RKR_{24}$) in the C-terminus, inducing membrane remodeling and disruption (Figure 11).

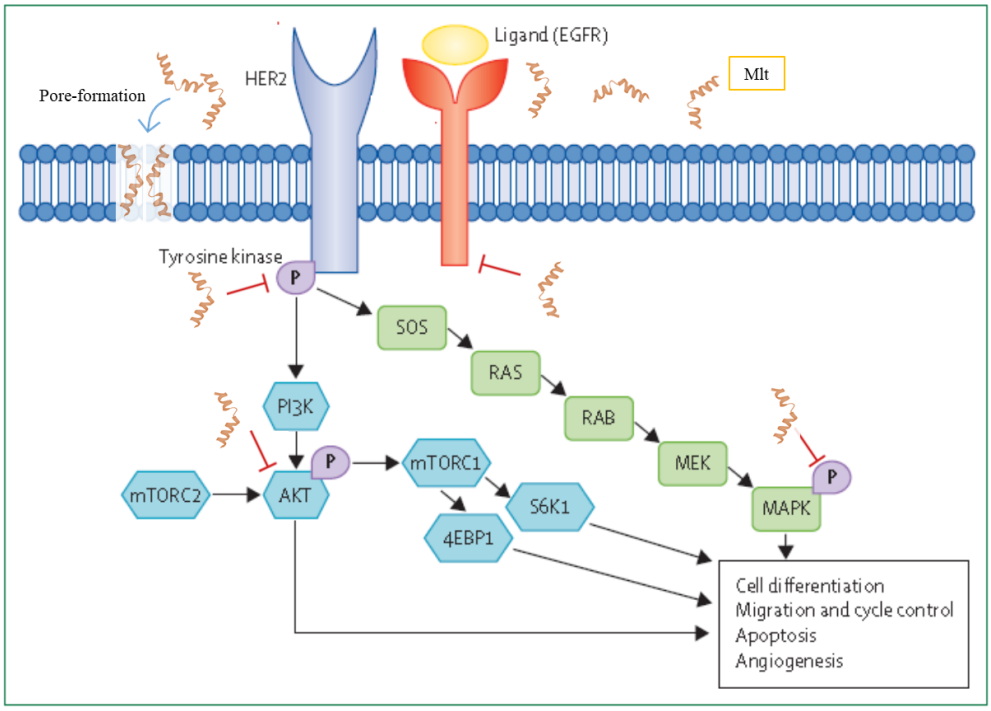


Figure 111. Schematic model of HBV and Mlt anticancer molecular mechanism in triple-negative and HER2-enriched breast cancer subtypes. Sketch inspired from (95).

While HBV and Mlt strongly downregulated the phosphorylation of the RTKs (p-HER2, p-EGFR, p-p44/42 MAPK, p-Akt, p-SAPK/JNK, and p-p38 MAPK) in HER2- enriched breast cancer (SKBR cells). They modulated the associated PI3K/Akt and MAPK pathways in a time-dependent manner. In TNBC (SUM159), HBV and Mlt strongly downregulated the p-EGFR phosphorylation. When Mlt was used alone, it suppressed p-Akt but upregulated p-p44/42 MAPK, p-SAPK/JNK, and p-p38 MAPK. In contrast, HBV upregulated p-p44/42 MAPK and p-Akt, as mentioned in Table 5. In their report, Duffy and coworkers claimed that the observed upregulation in TNBC may be due to the release of a negative regulatory feedback loop that triggers ERK signaling to protect the cell from apoptotic death (96).

Table 5. down- (┤) and up- (↑) regulation of protein kinases phosphorylation by HBV and Mlt

		Protein kinase phosphorylation					
		p-HER2	p-EGFR	p-p44/42 MAPK	p-Akt	p-SAPK/JNK	p-p38 MAPK
HBV	HER2BC	┤	┤	┤	┤	┤	┤
	TNBC	--	┤	↑↑	↑↑	--	--
Mlt	HER2BC	┤	┤	┤	┤	┤	┤
	TNBC	--	┤	↑↑	┤	↑↑	↑↑

11.1.1.1.2 Gastrointestinal cancers:

Mlt induced SGS-7901cell apoptosis (Human gastric cancer cells) via Cytochrome *c* signaling pathway activation. SGS-7901 cells stimulated by Mlt displayed typical apoptotic morphology. The occurrences' onset starts with a significant increase in ROS (reactive oxygen species) production after cells are exposed to Mlt treatment causing endometrial oxidative damage, mitochondrial lipide peroxidation, membrane fluidity reduction, and membrane lipide degradation. Hence the mitochondrial permeability was rendered irreversibly open, causing a decrease of the mitochondrial membrane potential Δψm.

These changes increase cytochrome c, AIF, and Endo G release and decrease the second mitochondrial-derived activator of caspases (Smac)/direct IPA binding protein with a low isoelectric point (DIABLO). Thus caspase-3 activation mediates the downstream effects that lead to the breakdown of cellular components and eventual cell death (97).

The Mlt molecular anticancer mechanism involved in HepG2 hepatocarcinoma consists of increasing p53 expression (pro-apoptotic protein) and decreasing Bcl-2 (survival protein) (87). Other studies showed the effectiveness of HBV and Mlt on other types of gastrointestinal cancer cells such as HepG2 hepatocarcinoma cell lines, HEP-2C human cervix carcinoma cell lines (larynx cancer), HCT116 human colorectal carcinoma cell lines, and Caco-2 human colorectal adenocarcinoma cell lines. The growth inhibition by HBV and Mlt

was dose-dependent with a height-significant cytotoxic activity on HepG2, HEP-2C, and HCT116. The IC_{50} were 1.26 μg/mL, 2.63 μg/mL, and 1.56 μg/mL, respectively (87,98,99).

11.1.1.1.3 Leukemia:

Only a few studies have been achieved to investigate HBV anticancer activity on leukemia cells. However, it has been demonstrated that HBV significantly decreases cell growth and viability, through cellular phenotypic changes and cell cycle disruption, in a dose-dependent manner in U937, HL60, K562, THP1 leukemia cells(2 to 3 μg/mL), and in acute lymphoblastic leukemia (CCRF-CEM) (78,100). HBV treatment increased the active forms (significant activity at 3 μg/mL HBV) of caspase-3, which partially plays a crucial role in HBV apoptotic death in U937 cells, caspase-9, and caspase-8. It resulted in a dose-dependent cleavage of PARP. HBV downregulated survival proteins such as Bcl-2, XIAP, and cIAP-2, but not cIAP-1. Unlike Bad's expression level, which was not altered, HBV dramatically increased the proapoptotic protein, Bax. Moreover, the Bax/Bcl-2 ratio increased in a dose-dependent manner when HBV-treated U937 cells. Furthermore, it was shown that HBV activated p-p38 and p-JNK. In contrast, the cell proliferation decrease was related to an ERK downregulation of upstream apoptosis induction, independent of the p38 and JNK pathways. Likewise, HBV downregulated the Akt signal pathway, which sensitized the HBV-induced apoptosis. COX-2 also seems to play a role in this process because it is known that COX-2 inhibition is correlated to an increase in apoptosis in several cancer models (101). HBV induced a specific COX-2 decrease (above 2 μg/mL HBV). Hence this downregulation inhibits cell growth and induces apoptosis, as depicted in the diagram in Figure 12. The same investigation also suggested that the HBV apoptotic effects in U937 cells are associated with Fas pathway upregulation. On the other hand, cumulated data demonstrated that HBV induced apoptosis through the downregulation of

human telomerase reverse transcriptase (hTERT). HBV molecular anticancer effects on U937 leukemia cells were attributed mainly to the main HBV peptide, Mlt (102).

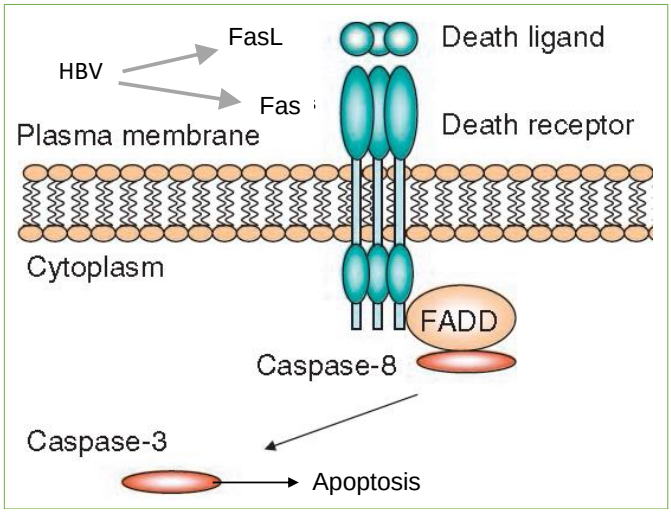


Figure 122. Schematic representation of HBV upregulation of the Fas signal pathway leading to death-inducing signaling complex (DISC) formation. The death domain (DD) of Fas recruits the adapter protein FADD. FADD, in turn, via its death effector domain (DED), recruits and activates caspase-8, which in turn triggers caspase-3 activation and apoptosis—inspired from (103).

Recent studies investigated the Mlt anticancer activity on other types of leukemia cells, including acute lymphoblastic leukemia (CCRF-CEM) and chronic myelogenous leukemia (K-562). It has been demonstrated that Mt could induce the apoptosis process via the intrinsic/mitochondrial pathway for the first time. Mlt triggered a dose-dependent cytotoxic effect and potent loss in mitochondrial transmembrane potential (a decrease in $\Delta\psi m$) after 24 and 48 h treatments of all tested leukemia cell lines tested concentrations. This depolarization leads to the release of apoptogenic factors and loss of oxidative phosphorylation, thus increasing the proteolytic activity of caspase-3 and caspase-7 in these cells (100).

11.1.1.1.4 Cervical carcinoma:

Several studies were focused on the HBV and Mlt molecular anticancer activity on different cervical carcinoma cells (CCCs). It has been shown that there were significant differences in CCCs sensitivity to HBV. In human cervical epidermoid carcinoma CaSki cells, HBV triggered a zymogen cascade resulting in morphological changes and decreased CaSki cell viability in a dose and time-dependent manner.

Treatment with HBV promotes ROS's potential production, increases Ca^{2+} into the cytosol, upregulates Bax, and decreases Bcl-2. These interactions decreased mitochondrial $\Delta\psi m$ and changes in the mitochondria outer membrane permeability, which permitted the release of the cytochrome *c*, AIF, Smac/DIABLO, and EndoG proapoptotic factors. The cytochrome *c* binds to phosphorylated caspase-9 and APAF-1 to form the apoptosome. This complex structure sparks caspase-3 phosphorylation and subsequently results in proteolytic cascade events, leading to DNA fragmentation and apoptosis (104,105).

HBV and Mlt inhibited cell growth, cell proliferation, and clonogenicity of HeLa cell lines in a dose and time-dependent manner (104,106,107). Results suggested that HBV inhibited DNA repairs and induced no necrosis or apoptosis in HeLa cells. The absence of those molecular mechanisms remains unexplained. However, he claimed that HBV exerts anticalmodulin activity and inhibits cell growth. This inhibition mechanism is unknown, but he supposed that it might hinder calmodulin-sensitive enzymes such as cyclic nucleotide phosphodiesterase. This inhibition appears to be mediated by forming a calcium-dependent high-affinity complex of calmodulin and Mlt (106).

The HBV anti-viral effects on CaSki, HeLa, C33A, and TC-1 cell lines were evaluated. HBV treatment resulted in significant cell growth suppression in HPV 16-infected cells (CaSki) and lesser suppression in HPV 18-infected cells

(HeLa). In contrast, the lowest suppression was observed in HPV-negative C33A. HBV decreased cell viability in a dose and time-dependent manner of all cell lines but with a significant difference in sensitivity between the three CCCs to this product. These outcomes were concordant with the decrease of the mRNA expression and protein levels of HPV16/18 E6 and E7 after HBV treatment. By 10 μg/mL HBV downregulated the mRNA expression levels of HPV16 E6 and E7 more significantly in CaSki than HeLa. Moreover, (91) demonstrated that Mlt inhibited MMP-9 (gelatinase-B) secretion, a metalloproteinase involved in the metastasis and called cancer migration (104).

11.1.1.1.5 Ovarian cancer:

Several studies evaluated the cytotoxic and proapoptotic effect of HBV and Mlt on many human ovarian cancer cell lines, including A2780cp cell lines (108,109), A2780, and A2780CR cell lines (110), SKOV3 (111–114), and AP-1 cell lines (112,115). HBV and Mlt induced cell death and reduction of cell viability in a dose and time-dependent manner. The suppression of cell growth was caused by apoptosis and necrosis (109). Generation of ROS and Bcl-2 downregulation was the anticancer molecular mechanism involved, triggering the intrinsic apoptosis pathway.

On the SKOV3 cell lines, Mlt inhibited the growth *in vitro*. The apoptotic mechanism contributed to the induction of death receptors, including overexpression of DR6 and DR3 and upregulation of death receptors proapoptotic proteins Bax, caspase-3 (an increase of cleaved caspase-3), and caspase-8. Likewise, Mlt inhibited the phosphorylation of the STAT/JAK2 pathway and also the expression of Bcl-2 (112,115).

In AP-1 Mlt induced programmed cell death by DR4 overexpression and cleaved caspase-8 (112,115).

11.1.1.1.6 Brain cancer:

Reports showed that Mlt, through 48 h and 3 μg/mL, significantly decreased proteases MMP-9 and ADAMTS-5 and TGF-β cytokines in U118 glioblastoma multiform cell lines, which are effective in cancer cell invasion (116).

11.1.1.1.7 Renal, bladder, and prostate cancer:

HBV and Mlt were investigated for their inhibitory effects on human renal cancer caki-1 cells. Findings showed that HBV and Mlt significantly suppressed PMA-induced invasion by inhibiting MMP-9 (gelatinase B) in a dose and time-dependent manner, a primary proteinase involved in the migratory metastasis processes. Furthermore, Mlt inhibited MMP-9 gene expression by blocking the PMA-stimulated activations of activator protein-1 (AP-1) and NF-κB by nuclear-translocating of *p*53 (a primary subunit of NF-κB) and c-Jun (a primary subunit of AP-1). It also suppressed the PMA-induced phosphorylations of ERK and JNK/MAPK, upstream factors implicated in AP-1 and NF-κB, two major promoters of MMP-9 gene expression (91). In human bladder cancer and TSGH-8301 cells, HBV induced apoptosis by promoting cell morphological changes and decreasing cell viability by inducing apoptosis, mediated by caspase-dependent independent death pathway and ER stress in TSGH-8301. HBV upregulated the protein levels of Bax, caspase-8, caspase-3, and caspase-9 phosphorylation, Endo G, PARP, Fas, FasL, and TRAIL, which caused nuclear condensation and formation of apoptotic bodies. At the same time, it downregulated the protein levels of Bcl-2, Bid, and XIAP in TSGH-8301 cells. HBV also increased the ER release of Ca^{2+} hance Δψm loss, and the ER stress transducers levels, including PERK, ATF-6α, ATF-6β, IRE-1α, GRP78, GADD153, XBP-1 caspase-4. Likewise, HBV increases the release of Ca^{2+} and hence Δψm loss (117).

Anticancer effects of HBV and Mlt on human prostate cancer LNCaP, DU145, and PC-3 also were investigated. HBV and Mlt induced apoptotic cell death in these cell lines by triggering antiapoptotic proteins' decrease and promoting proapoptotic once. This molecular mechanism is mediated by suppressing constitutively activated NF-κB through the inhibition of IκB phosphorylation and the inhibition induction of nuclear translocation of p50 and p65 subunit in PC-3. Moreover. BV (3-6 mg/Kg) administration to nude mise implanted with PC-3 cells results in the inhibition of tumor growth and activity of NF-κB accompanied by apoptotic cell death (118).

11.1.1.2 Anti-inflammatory activity:

It is well known that HBV and its main peptides Mlt and apamin exhibit anti-inflammatory activities. HBV and Mlt induced inhibitory effects on LPS-induced expression of COX-2, cPLA2, iNOS, generation of the PGE2, and NO (119–121). Moreover, HBV and Mlt prevented LPS-induced transcriptional and DNA binding activity of NF-κB via the inhibition of IκB release and p50 translocation. The molecular mechanism of the anti-inflammatory of HBV was pinpointed. This matrix significantly decreases the MAPK mRNA in a dose-dependent manner (122). Likewise, HBV suppressed TNF-α pro-inflammatory cytokine production (123). In LPS-stimulated BV2 microglia, HBV and Mlt significantly inhibited NF-κB, JNK, and Akt pathways. They significantly inhibited the proinflammatory cytokines (IL-1β, IL-6, and TNF-α) (121). Besides, Mlt inhibited the expression of proinflammatory cytokines by suppressing the activation of the NF-κB signaling pathway, ERK, and Akt (124). HBV prevents the development of antigen-induced arthritis in rabbits through endogenous glucocorticoid action (125). Furthermore, HBV and apamin inhibit proinflammatory cytokine production and inflammasome formation in a murine model of gouty arthritis (34).

The HBV anti-inflammatory action is closely dose-dependent. With a high dose of HBV (20 mg/Kg) IL-1β, IL-6, TNF-α, TGF-β1, and IFN-γ, DNA damage levels showed significant levels, and MPO activities were significantly higher. But at a low dose of HBV administration (4mg/Kg), all those parameters significantly decreased, excluding IFN-γ, whose level increased (15).

11.1.1.3 Other therapeutic potential of HBV:

HBV and their main components Mlt, apamin, and PLA2 proved to have many other activities and therapeutic potential, including antioxidant activity (126–129), antimicrobial and antiviral activities (16,104,130–132), antinociceptive activity (79), antipsychotic activity (133), a benefit on the reproductive performance and immune response (17). HBV and its peptides showed efficiency in several experimental and clinical pathologies such as arthritis (79,134), Parkinson's disease (135–141), acetylsalicylic acid-induced gastric ulceration (142), muscle injury treatment (28), diabetic wound healing (143,144), Bertter syndrome, hypertension, congestive failure, and atrial fibrillation (48), atherosclerosis lesion and restenosis (145), and recently in control of SARS-CoV infections (16).

12 Melanoma skin cancer :

12.1 Definition:

Melanoma is an aggressive and the deadliest form of skin cancer that generally starts in the skin cells called melanocytes or the cells that produce melanin. These are the cells that darken when exposed to the sun, a protective response to shield the skin's deeper layer from the harmful effects of UV rays. Skin melanomas can strike anyone but are most common in people with pale skin, getting more common. Skin melanoma is less common than some other types of skin cancers. However, melanoma is more dangerous because it is much more

likely to spread to other parts of the body if not caught and treated early. It usually starts in a mole and is strongly linked with UVA exposure and UVB radiation from the sun or sunbeds. However, it can occur in any tissue that contains melanocytes, such as the eye or the intestines. Genetic factors can also increase the risk of melanoma. At diagnosis, melanoma has a numerical stage based on how deeply it has grown into the skin and spread to other parts of the body (146–148).

Most skin cancers start in the top layer of skin, the epidermis. This latter is separated from the deeper layers of skin by the basement membrane. When skin cancer becomes more advanced, it grows through this barrier and into the deeper layer (149).

There are four stages of skin melanoma, According to the American Joint Committee on Cancer (AJCC), as explained in Figures 13 and 14 (150):

Stage 0: in this earliest stage, the melanoma cells are still confined to the epidermis, the top layer of the skin.

Stage 1: At this stage, the melanoma can be up to 2mm thick and may or not be ulcerated, but there is no sign of it spreading to lymph nodes. It can be easily removed by surgery.

Stage 2: The melanoma is up to 4mm thick by this stage, but there is still no sign of spread to nearby lymph nodes or other sites. Most stage 2 melanomas can still be treated with surgery.

Stage 3: cancer cells have now spread deeper into the skin, lymph vessels, or nearby lymph glands. More than half of stage 3 melanomas come back after surgery.

Stage 4: By this advanced stage, cancer has spread to other parts of the body, such as the lungs, liver, brain, bones, distant lymph nodes, or other areas of the skin. Treatment depends on which areas are affected.

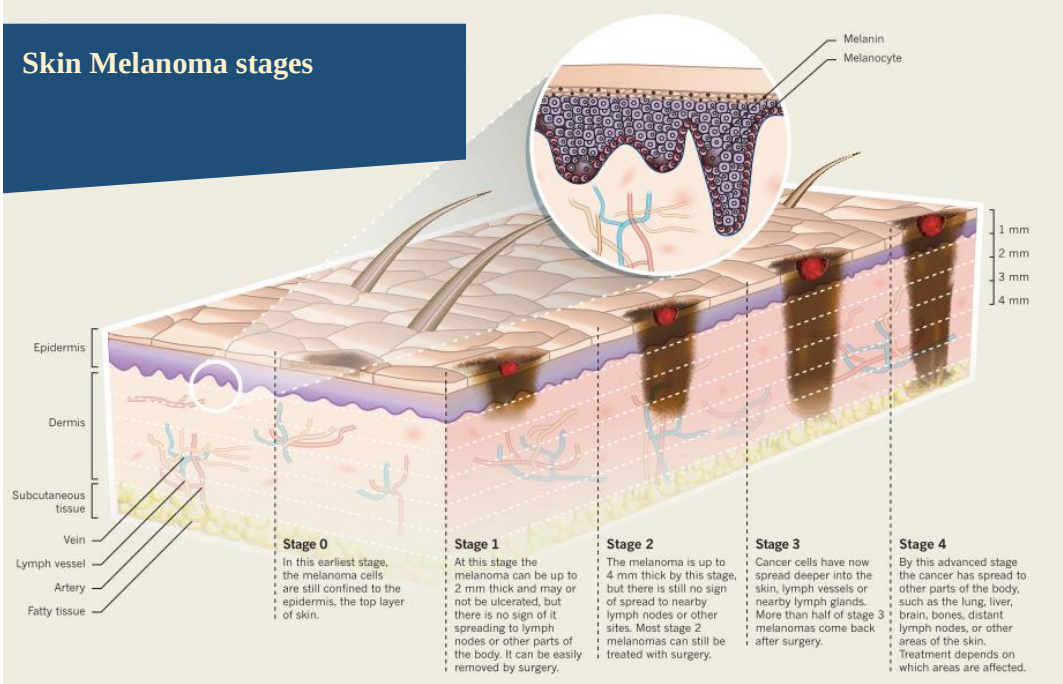


Figure 133. The four stages of skin melanoma.

12.2 Epidemiology:

Cutaneous melanoma accounts for 75% of skin cancer-related deaths, and the incidence has been increasing worldwide (151–153). Nearly 300,000 new cutaneous melanoma cases are reported each year worldwide, with a high increase in incidence among young people (153). According to the American Cancer Society (ACS), it is estimated that the number of new melanoma cases diagnosed in 2021 will increase by 5.8%, and the number of melanoma deaths is expected to increase by 4.8%. An estimated 207,390 cases of melanoma will be diagnosed in the U.S. Of those, 106,280 patients will be invasive, penetrating the epidermis into the skin's second layer, the dermis. Of the invasive cases, 2,260 will be men, and 43,850 will be women (154). Figure 15 shows the U.S., where melanoma is now the fifth most common cancer form (146).

Unfortunately, no skin cancer data covering the whole of Morocco is available except for certain studies collected from a few medical theses. 91 cases of cutaneous melanoma were recorded between 1984 and 2004 at the CHU IBN

ROCHD in Casablanca, giving an average annual rate of 4.33 cases per year. Another study carried out at the IBN SINA University Hospital in Rabat between 1994 and 2005 reports an average annual rate of 2.5 cases per year, with a marked increase in melanoma incidence during these years (155).

55 cases of cutaneous melanoma have been listed between 2000 and 2007 in Agadir and regions(156), and 60 patients were recorded from 2006 to 2010 in Casablanca and areas (157).

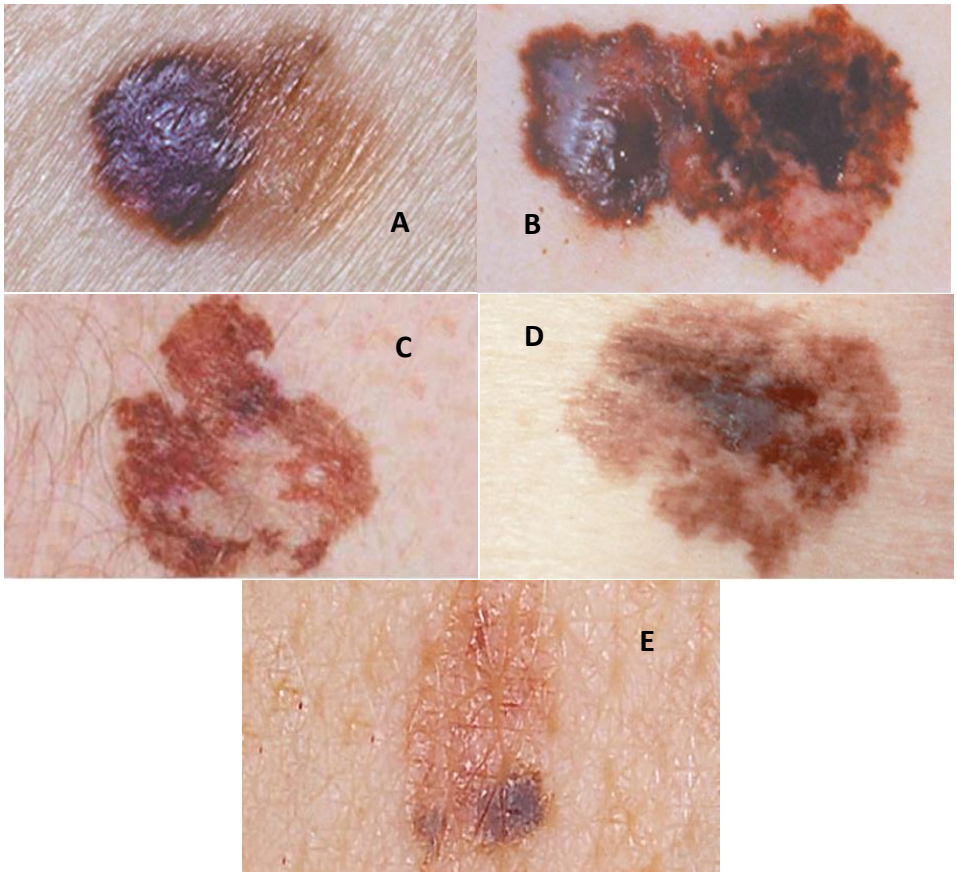


Figure 144. The ABCDE melanoma guide according to the AAD where (A) for asymmetry, the shape of one half does not much the other; (B) for the border, the edges are ragged and blurred; (C) for the color is uneven and may include shades of black, brown, and tan; (D) for diameter, there is a change in size, usually an increase; (E) for evolving, the mole has changed over the past few weeks or months.

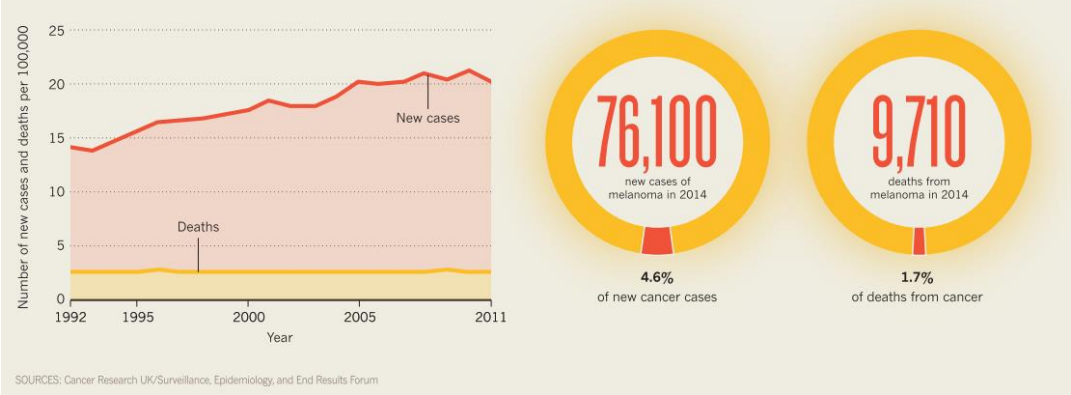


Figure 155. The increasing burden of skin melanoma in the U.S.A.

12.3 Causes, risk factors, and prevention:

Risk factors for melanoma include both intrinsic (genetic and phenotype) and extrinsic (environmental or exposure) factors: sun exposure, pigmentary characteristics, multiple nevi, family and personal history of melanoma, immunosuppression, and environmental exposure (148,158).

One of the most important tools to prevent skin cancer is avoiding sunburn by minimizing sun exposure when the UV index is 3 or above, especially in the middle of the day when UV levels are more intense. Seeking shade, wearing a hat covering the head, neck, and ears, wearing sun-protective clothing and close-fitting sunglasses, wearing an SPF30 sunscreen, and finally avoiding solariums (158).

12.4 Early detection and diagnosis:

Diagnosing melanoma begins with a visual examination. Often the first sign of melanoma is a change in size, shape, and color. Or feel of a mole. To make it more common for the people, the American Academy of Dermatology (AAD) published the ABCDE guide to determine if an unusual mole or suspicious spot on the skin may be a melanoma (159–161). However, studies show that distinguishing between benign pigmented lesions and early melanomas can be complex, and even experienced dermatopathologists can have differing opinions

(162,163). For this, it is well established that prognosis is related to the stage of disease at diagnosis, making early detection critical (164). In addition to physical examination, biopsy comes the first technique range of melanoma diagnosis; however, the punch and shave biopsy leads to increased microstagin inaccuracy (165). Ten different non-invasive techniques could be employed for skin and cutaneous melanoma, including dermoscopy, sequential digital dermoscopy, total body photography, computer-aided multispectral digital analysis, electrical impedance spectroscopy, Raman spectroscopy, reflectance confocal microscopy, multiphoton tomography, stepwise two-photon-laser spectroscopy, and quantitative dynamic infrared imaging. Unluckily, none of these techniques can provide a definite and final diagnosis or completely replace the histopathological examination (166).

12.5 Melanoma skin cancer treatments:

The treatment choice for cutaneous melanoma depends on the disease's stage, the location of the tumor, and the overall patient health. Five types of standard treatment are used (167–169):

- Surgery to remove the tumor is the primary treatment for all stages of melanoma.
- Chemotherapy; uses drugs to stop the growth of the cancer cells, either by killing the cells or by stopping them from dividing.
- Radiotherapy
- Immunotherapy
    1. immune checkpoint inhibitor therapy, including CTLA-4 inhibitor therapy and PD-1 and PD-L1 inhibitor therapy
    2. interleuin-2 (IL-2) therapy
    3. Tumor necrosis factor (TNF) therapy
- Targeted therapy:

1. Signal-transduction inhibitors therapy, including BRAF inhibitors (dabrafenib, vemurafenib, encorafenib), block the activity of proteins made by mutant BRAF genes MEK inhibitors (trametinib, cobimetinib, binimetinib) that block MEK1 and MEK2 which affect the growth and survival of cancer cells.
2. Oncolytic virus therapy; uses a virus that infects and breaks down cancer cells but not normal cells.
3. Angioinhibitors are types of targeted therapy that block the growth of new vessels.

- New types of treatment are being tested in clinical trials.

12.6 Contribution of HBV in melanoma skin cancer treatment:

Several studies proved the anticancer activities of HBV and its main components Mlt, apamin, and PLA2. Melanoma accounts for only about 1% of skin cancers but causes many skin cancer deaths (146). In a prior study, Mlt inhibits the melanotropin receptor in M2R melanoma cell membranes (170). Besides, HBV inhibits the proliferation of melanoma K1735M2 cells *in vitro* and B16 melanoma, a transplantable solid melanoma in C57BL/6 mice, *in vivo*. The inhibition was in a concentration and time-dependent manner and occurred by the cell cycle arrest at the G1 stage. HBV induced apoptosis-like cell death as identified by DNA fragmentation. In the *in vivo* study, HBV inhibited the solid tumor in mice 24 h after being inoculated with B16 cells (171). On the other hand, Mlt induced apoptotic cell death in human melanoma A2058 cells in a caspase-independent manner through ROS level increase and subsequent disruption $\Delta\psi m$ transition through the release from the ER of Ca^{2+}. This potential disruption leads to the release of AIF and EndoG protean into the nucleus, triggering the apoptosis cascade process (172).

Chapter II: Review of analytical methods for honey bee venom characterization.

1 Background:

Many methods have been described for bee venom characterization, including biological tests, chemical approaches based on typical protein reactions, and separation techniques (173). Additionally, many factors impact the choice of the suitable method, including the non-proportionality of bee venom components. In some cases, melittin and PLA2 masked the detection of less abundant ingredients (13). Consequently, the purification of trace substances by conventional techniques seemed very far from desirable results (174). Unluckily, no reliable or unified methods can be considered the basic process that leads to a standard product (175). On the other hand, The purification of honey bee venom components is still far from easy, partly because most of the bioactive ingredients are highly basic and of similar molecular weight and partially because they are contained initially in a highly complex mixture (176). Table 6 outlines most analytical methods used for the main bee venom proteins, peptides, amino acids, and trace substances analyses.

2 HBV collection techniques:

Collecting fresh bee venom or apitoxin requires careful work with the highest degree of cleanliness, mainly if it occurs in the apiary. The technique of bee venom extraction depends on the required purposes. We can collect HBV from a single honey bee by gently squeezing the venom sac with a glass capillary. Or centrifugation of the blend obtained by repeated puncturing; with a sharp needle of both sac and gland and recovering the supernatant for the expected analysis. (177–179).

Table 6. The most important analytical techniques used for the main Proteins, peptides, and amino acids of HBV.

Techniques		Identification of the technique	Substance analyzed	Material employed	References
Biochemical techniques	Enzyme assays	Phospholipase essay	PLA2 & isoforms	BVG	(177,179,180)
		Melittin essay	melittin	BVG	(179)
		Allergosorbent essay	PLA2 & isoforms	BVG	(178)
	Electrophoresis	SDS-PAGE	PLA2 & isoforms + melittin	CBV	(173,177,181)
		Propionate acid/urea PAGE	melittin + apamin + MCDP	DBV	(182)
		Isoelectric focusing technique	PLA2 & isoforms + melittin	BVG	(179)
	Allergosorbent methods	Titrimetric & Allergosorbent method	PLA2 & isoforms	BVG	(178)
Chromatography techniques		HPLC	PLA2; hyaluronidase; melittin; apamin; MCDP	CBV	(183–186)
Spectrometry techniques		Liquide scintillation counting (LSS)	PLA2 & isoforms	BVG	(177)
		Fluorescence spectrometry	melittin; apamin; MCDP	BVG	(174,187)
Coupled techniques		HPLC & mass spectrometry	melittin; apamin; MCDP; secapin; histamine; free amino acid; PLA2 &isoforms	BVG & CBV	(20,187–193)
Spectroscopy techniques		Infrared spectroscopy	biologically active components of HBV	CBV	(194)
Voltammetry techniques		Square wave stripping Voltammetry	melittin	CBV	(195)

Or, merely, the stings can be removed directly from the individual bee without anesthesia (55,196,197) or anesthetizing on ice (198).

Several collector devices have been made for collecting bee venom. Benton et al., 1963 developed a wood frame with metallic wires and a glass plate covered tightly with a nylon sheet. 33 Volts were applied for 5 min as a stimulating tension. The device was fitted underneath the broad chamber of the colony (199). Gunnison., 1966, introduced a cooling system into a similar device to Benton's (Gunnison, 1966) to enhance the collection's yield and reduce bee mortality, Figure 16. As for Rybak, he conducted two works. The first is to optimize the position of the venom-collecting frames in the beehive (200). In their second research, they coupled two stimulating factors: electrical and sound stimuli, Figure 17. Therefore, they claimed that the best position consists of placing two frames at the second outermost opposite ends of the beehive. Also, coupling both stimulating factors increases bee venom yield (201).

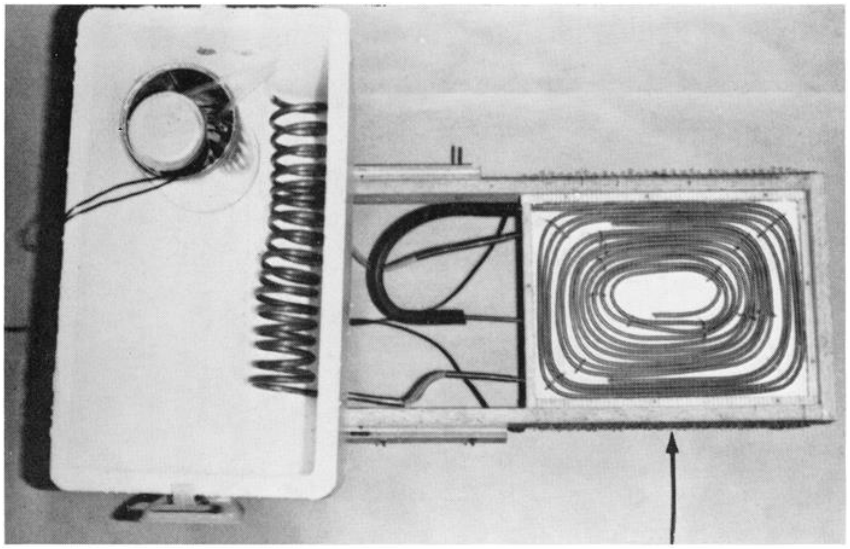


Figure 166. Modified Cornel venom collector. The arrow points to the collector with an underling copper coil. The box on the left contains the cooling coil and dry ice.

Figure 177. A set of equipment used for venom collection using combined electrical and sound stimulus.

3 Analytical techniques of HBV characterization:

3.1 Peptides and proteins Characterization:

3.1.1 Separation and purification techniques:

Gel filtration and thin-layer chromatography were essential techniques for the purification, separation, and characterization of HBV peptides and proteins (enzymes). Habermann was the first to describe these techniques. Many works have focused on the number of purification steps, the pH and eluent nature, temperature conditions, and gel filtration resin types (53,176,202–204). By achieving many tandem columns of Sephadex G-25 and Sephadex G-50 using 2% acetic acid, melittin, MCDP, and apamin have been separated and purified. However, there was considerable overlapping of the profile of these three peptides.

Moreover, it was impossible to estimate the relative amount of peptides present. Yet, a subsequent step of purification of the resulted fraction by SP-Sephadex C25 using ammonium acetate/acetic acid buffer leads to the isolating of three new peptides: melittin-F, tertiapin, and secapin (176). Before that, melittin and

minimine were isolated and purified melittin and purified minimine by performing a similar protocol using methanol and acetone extraction followed by polyacrylamide, Sephadex gels, and anion exchangers (53). Nonetheless, such a procedure was extremely tedious, time and sample-consuming, incidentally wasteful for materials, and not suitable for quality control purposes or qualitative essays (174,176,205).

Other alternative strategies were used without preliminary purification steps. A single chromatographic step through columns of Heparin Sepharose CL-6P at pH 6.8 using a salt gradient with a high concentration was used to elute melittin once it bounds to the column. The separation was performed by thin-layer chromatography and the identification by fluorescence spectrometry. The heparin's feature to precipitate melittin and MCDP made this protocol the technique of choice to purify apamin at a satisfying degree (174). Propionic acid/urea polyacrylamide gel electrophoresis combined with gel filtration was employed to identify PLA2 and purify melittin, apamin, and MCDP to homogeneity. This method upgraded the quality of the electrophoretic separation, reproducibility, and robustness of the analysis. However, it cannot be used to estimate the molecular weight of peptides (206). Thin-layer isoelectric focusing polyacrylamide gels have allowed the identification of considerable PLA2 variabilities between European and Africanized honey bees with a variation coefficient of 47.1% and 27.1%, respectively (179). Besides, the use of titrimetric assay of PLA2 activity showed significant PLA2 age-to-age differences with a minimum level at the eclosion and a maximum level (43 μg) in bees one week to 10 days old (178). Variability also concerns castes; thus, queen bees contain less than one-quarter of phospholipase activity in venom from worker bees (177). HBV Hya has been described and purified using a complex multistep procedure founded on repetition and alternation of gel filtration and ion-exchange chromatography and achieved by an affinity-purified rabbit anti-PLA2

immunosorbent column. The product showed a specific activity of 1600 u/mg and was stable to different denaturing agents (207). HBV Hya was also determined by an indirect method based on incubating HBV without any sample preparation with a known amount of hyaluronic acid followed by CE analysis to monitor the peak representing hyaluronic acid (208).

3.1.2 Electrophoresis and chromatography techniques:

Capillary zone electrophoresis (CZE) has been validated and used for the melittin and PLA2 determination in HBV. The technique was reliable, fast, performant, and inexpensive (209). High-performance capillary zone electrophoresis (HPCZE) was developed and validated, employing an internal standard (IS). MCDP, apamin, PLA2, and melittin were detected with high precision and accuracy. Tertiapin also was eluted but was not quantified because it is a trace peptide (190).

High-performance liquid chromatography (HPLC) techniques took a broad interest from researchers. Hence, various designs have been upgraded to identify and quantify HBV peptides and proteins. Many methods were submitted to the validation procedure for quality control purposes, fingerprinting, and standardization of the product; Table 7. Exclusion high-performance liquid chromatography (SEC-HPLC) on the I-25 column under the isocratic condition was used to identify MCDP, apamin, PLA2, melittin, and Hya. But because of the melittin/apamin ratio (30:1), the chemical behavior, and their similarities, reverse-phase-HPLC (RP-HPLC) on LiChrospher Si 100, RP-18 column under the same elution conditions has been used to determine apamin alone and deduce melittin quantity by subtracting [(Melittin + apamin) – apamin] (205). The use of a linear gradient after a preparative column on Sephadex G50, the RP-HPLC run has shown a good separation of procamine, apamin, MCDP, tertiapin, melittin-F, secapin, PLA2, Hya, and melittin (183). Under linear-gradient and IS employment, the validation of an RP-HPLC has been shown

good precision and accuracy for identifying and simultaneous determination of the major constituents of HBV, which allows using this method for HBV standardization (210). Moreover, a validated RP-HPLC combined with intrinsic fluorescence detection (IFD)for melittin quantification in individual bee venom glands (BVG) was rapid (Rt = 6.82 min), more selective, and sensitive (187).

Table 7. Column types, elution gradients, and detection systems for the main HPLC designs used for identifying and purifying HBV peptides.

Chromatography technique	Column type and elution conditions	Detection system	Substances identified and purified	References
SEC-HPLC RP-HPLC	I-125/ isocratic LiChrospher Si 100, RP-18/isocratic	DAD	Melittin, apamin, MCDP	(205)
RP-HPLC	Hypersil WP-300 C18/linear gradient	UVD	Melittin, apamin, MCDP, melittin-F	(183)
RP-HPLC	C18;100, 180, 300 Å/linear gradient	UVD	Melittin, apamin	(175)
RP-HPLC	Synchropack C8/ linear gradient	DAD	Melittin, apamin, MCDP	(210)
RP-HPLC	Sepax Bio-C18	IFD	Melittin	(187)
RP-HPLC	XSelect CSH130 C18/linear gradient	DAD	Melittin, apamin	(129)

3.1.3 Spectrometric techniques:

Several spectrometry techniques have been employed for HBV characterization. The determination of accurate molecular mass, the detection of chemical compounds at great sensitivity with a shorter sample run, a high selectivity, specificity, and a typical limit of detection are more than enough to deal with most analytical problems encountered with previous methods. Mass spectrometry has become the key analytical tool for HBV analysis. Multiple platforms with distinct capabilities are used for sample analysis, such as matrix-

assisted laser desorption ionization-time of flight (MALDI-TOF), electrospray ionization-liquid chromatography/mass spectrometry (LC-ESI-/MS), ion trap instruments, and Fourier transform mass spectrometry (FT-MS) systems. (129,191,192,211–213). A triple quadrupole tandem mass spectrometer coupled to an ESI interface using multiple reaction monitoring (MRM) was used to determine melittin and apamin simultaneously. It has been demonstrated that this technique could be applied to monitor HBV compounds and their stability over time. Quintuply charged molecular ions with *m/z* 570.2 $[M+5H]^{5+}$ and quadruply charged molecular ions with *m/z* 507.7 $[M+4H]^{4+}$ were precursor ions for melittin and apamin, respectively. N-terminal $b3^{5+}$ (*m/z* 85.9), and $b5^{5+}$ (m/z 142.7) ions, as well as $b8^{5+}$ (m/z 507.7 → 198.9), and $y3^{3+}$ (m/z 507.7 → 110) MRM transitions were chosen for qualification and quantification of melittin and apamin, respectively (189).

Two complementary mass spectrometry platforms with distinct capabilities MALDI-TOF-MS and nano-electrospray ionization quadrupole time of flight mass spectrometry (nanoESI-QqTOF-MS), were combined to develop a particular HBV characterization process. The use of an IS (glucagon-like peptide) and two different matrices: sinapinic acid and α-cyano-4-hydroxycinnamic acid (CHCA), allowed good precision and accuracy and ensured a wide variety of MALDI-TOF-MS analyses. Apamin and MCDP were detected by using CHCA, while they were not found by sinapinic acid. By this latter, four degradation products and precursors of melittin were detected. Using nanoESI-MS experiments, many peptides were identified, including melittin and its breakdown products, apamin, MCDP, secapin, and one small peptide (HTGAVLAGV, 822.53 Da) (190). It has been shown that a similar semi-quantitative MS (MALDI-TOF-MS) proved helpful for the standardization, screening, and protein fingerprinting of this matrix (191). ESI-MS^n, nanoESI-MS^n, and ESI-QTOF-MS combined with HPLC among various platforms have

been employed to characterize PLA2, Hya, MCDP, adolapin, apamin, and melittin (129,213). HBV compounds in envenomed tissues have also been monitored by direct MALDI-TOF-MS and liquid chromatography-electrospray ionization high-resolution mass spectrometry (LC-ESI-HRMS). MALDI mass spectrometry imaging (MSI) was used to map, over time, the diffusion and distribution of PLA2 among three other essential components (214). Another report, using nanoHPLC-nanoESI-MS, showed variations in melittin and PLA2 under particular climatic, seasonal, and feeding factors. A progressive rise of PLA2 p roduction was evidenced from January, reaching a maximum in Jun and a period of decrease, achieving a minimum in December (215). Hya appeared by mass spectrometry techniques simultaneously with most peptides and proteins in HBV. Electrospray ionization linear trap Fourier transform cyclotron resonance mass spectroscopy (ESI-LTQ-FT-ICR-MS), MALDI-TOF/TOF, ESI–QTOF, as well as other approaches, can be used for its characterization (20,192,216–218).

3.1.4 Spectroscopic techniques:

Several spectroscopic analyses have been used to explain the structural relationship and the activity of the main HBV peptides, including melittin, apamin, and tertiapin. It concerned structural aspects of HBV peptides in solution at different temperature conditions. The three-dimensional structure of the tetrameric melittin was determined by X-ray crystallography analysis at 2 and 6 Å resolution. The X-ray diffraction patterns indicated that melittin tetramer contains at least a 2-fold axis of rotation (219). The exploration of melittin thermodynamic using circular dichroism spectroscopy (CD) showed that this peptide converts from monomeric random coil to α-helical tetramer when pH is raised from 4.0 to 9.5, ionic strength is increased, and the temperature is raised or lowered 37°C, or when phosphate is added (220–222). A novel X-ray absolute-scale refinement method was used to provide insight into

melittin penetration into the hydrocarbon core of bilayer membranes, explaining the lytic action of α-helical melittin. It has been found that the helical axis is aligned parallel to the bilayer plane at a depth of glycerol groups. The crucial initial step toward membrane lysis results from the self-association of amphipathic helices (223). Proton nuclear magnetic resonance spectroscopy (^{1}HNMR) was employed to understand melittin behavior at different temperatures (heat denaturation), pH, and ionic strength. It has been demonstrated that melittin assumes an amphiphilic spatial structure, which is stabilized by forming mixed micelles in an ionic solution or self-aggregation (224). Likewise, melittin undergoes a conformational transition from monomer to tetramer to monomer by elevating temperature from 20 to 70°C. The tetramer has maximum conformation stability at around 20°C. Melittin, including a trans proline bond, participates in the transition, whereas melittin, including a cis proline, does not (225). Using two-dimensional nuclear magnetic resonance spectroscopy (NMR), apamin structure in solution displayed an α-helical core (residues 9-15) with fraying form residues 16-18, and a β-turn involving residues 3-5 and a nonstandard turn including 6-8 (32,226). However, by using distance geometry derived from NMR, some differences were described, which the most relevant in the loop between β-turn (residues 2-5) and C-terminal α-helix (residues 9-17) (227). As for tertiapin, it showed similarities in the secondary structure of melittin and apamin but displayed different tertiary structures, by using CD and NMR (46).

There are not enough spectroscopic studies available for HBV enzymes. X-ray diffraction, molecular dynamics, and NMR allowed intriguing secondary and tertiary structural information on PLA2 (15-16 KDa) (228,229). Other methods can be used to determine the primary structure, including tandem MS and bioinformatic methods where the protein sequence is derived from complementary DNA (cDNA) or messenger RNA (mRNA) or a DNA sequence

in databases (230). X-ray crystallography was employed to determine the crystal structure of HBV Hya. It is defined in two different crystal forms. The complex with an Hya oligomer provides insight into the mode of substrate binding and the catalytic mechanism. This enzyme shares more than 50% sequence identity with hyaluronidase from other Hymenoptera and 30% of several mammalian enzyme identities (67,68).

3.1.5 Voltammetric determination of melittin in honey bee venom:

Thanks to its low cost, the short time required for analysis, and the possibility to perform analysis without extraction or pre-concentration, voltammetric techniques were also used. A square wave stripping voltammetry (SWSV) method was developed and validated using a gold electrode for indirect determination of melittin in non-aqueous medium lithium perchlorate ($LiClO_4$) in dimethyl sulfoxide (DMSO) and tetrabutylammonium tetrafluoroborate (TBATFB) in acetonitrile (MeCN). The determination of melittin is deducted since there is a proportionality between the decrease of the oxidation peak current of ferrocene at 110 mV and the increase of melittin amount (195).

3.2 Omics:

HBV has shown enormous potentialities, making it an attractive target for prospective pharmaceutical discovery (216). Several omics technologies approaches have been employed to identify a wide range of protein and non-protein-peptide components at very low concentrations. Hence demonstrates new HBV components with allergic potential, a better understanding of the physiological and pharmacological role of the whole compounds, study of the protein-protein interaction at a cellular level, and identifies the mechanism that protects the venom gland secretory cells against this harmful substrate (231–233). One of the most critical issues in proteomics has always been the difficulty of accessing minor or underrepresented proteins in a complex sample, let alone

the considerable complexity of HBV. Two MS-based approaches were used for global quantitative protein profiling. Shotgun proteomic approaches (SGPA) based on isotope tags to label proteins, followed by a phase of separation by multidimensional liquid chromatography and mass spectrometry analysis. The second proteomic approach, the classical one, is founded on two-dimensional electrophoresis (2-DE) mass spectrometry. One-dimensional electrophoresis can be employed (233–236). This latter seems to lack sensitivity to detect additional venom compounds; besides, it excludes proteins of low abundance, high hydrophobicity, extreme isoelectric point (*pI* <4 or *pI* >10), and *Mr(<15kDa or >200kDa)* (216,236). Peiren et al., 2005 identified three new proteins using the classical approach by applying two mass spectrometry analysis strategies. MALDI-TOF/TOF-MS and nano-HPLC-MS/MS. The first protein has a platelet-derived and a vascular endothelial growth factor family domain. The second shows no homologies with any known protein and the third exhibits similarities with major royal jelly protein 8 (231). Likewise, using the same strategies, Peiren's team revealed four antioxidant enzymes responsible for the protection of honey bee venom gland secretory cells: CuZn Superoxide dismutase (SOD1), glutathione-S-transferase sigma-1 isoform A (GSTS1), peroxiredoxin 2540 (PXR2540), and thioredoxin peroxidase-1 isoform A (TPX1) (232). Using a strategy based on the shotgun proteomic approach and the sample enrichment employing a combinatorial peptide ligand library (CPLL), 102 proteins were identified, 83 among them described as new ones. Thirty-three putative toxins belong to esterase, protease inhibitors, carbohydrate degrading enzymes, growth factors, major royal jelly proteins (MRJP), and anti-microbial peptides (236). Using SGPA and label-free quantification, a comparative investigation studied proteome and phosphoproteome of Africanized honey bees with *Apis mellifera ligustica* and *Apis mellifera carnica*. 51 proteins have been identified; 42 were shared among all three venoms, and

many previously unreported. By this proteomic investigation, two phosphoproteins; melittin and icarapin, were shown. In addition, the basic toxicological tests revealed that phosphorylation reduces melittin activity compared to the native peptide as well (216). Applying different enrichment strategies CPLL and solid-phase extraction, the use of SGPA evidenced 4 new hypothetical proteins (192). The outcome was much more promising by combining two platforms analysis: LC-MALDI-TOF/TOF-MS and LC-ESI-QTOF-MS. 269 proteins were detected, 49 were honey bee toxins belonging to venom enzyme classes: esterases, proteases/peptidases, protease inhibitors, MRJP, and 5 were additional putative toxins (212). Recently, seven low-molecular-weight organic acids in HBV using an MS-based methodology were determined. Two protocols for sample preparation were employed. A solid-phase extraction to determine malonic acid, fumaric acid, glutaric acid, and kynurenic acid. A dilute-and-shoot method for the determination of citric acid, malic acid, and succinic acid. Citric acid was the most abundant organic acid in HBV, with 99% of the sum of the whole organic acids determined (237). The total metabolites in HBV was identified and quantified by applying three different mass spectrometry (MS)-based methodologies. A high-resolution, accurate-LC-Q-Orbitrap-MS untargeted metabolomics analysis for the first approach allowed a broad overview of HBV metabolomic compositions. Metabolites belonging to amino acids (1-aminocyclopropanecarboxylic acid, 3-aminoisobutanoic acid), catecholamines (3-O-methyldopa), organic acids (citric acid, fumaric acid, glycolic acid), carbohydrates (gluconic acid, glucosamine, glucuronic acid/galacturonic acid, glyceraldehyde, glyceric acid, tartaric acid), purines (adenine, hypoxanthine, uric acid, xanthine), pyridines (nicotinic acid, pyridoxal), pyrimidines (5-methylcytosine, cytosine, dihydrouracil, thymine, uracil), nucleosides (cytidine, guanosine, uridine), and nucleotides (adenosine 5′-monophosphate, uridine 5′-monophosphate) were detected and identified.

43% of all metabolites found in HBV were amino acids and derivatives. Two targeted metabolomics approaches were afterwards performed. A wide-spectrum metabolite determination using Absolute IDQ p180 kit and LC-QqQ-MS/MS, which allows for simulataneouss quantifaication of 188 metabolites, including acylcarnitines, amino acids, biogenic amines, hexoses, glycerophospholipids, and sphingolipids. A wide-spectrum amino acid determination using aTRAQ kit and LC-QqQ-MS/MS, which allows for simultaneous quantitative analysis of 42 analytes , including proteogenic amino acids, non-proteogenic amino acids, and their derivatives (193).

3.3 Volatiles:

Three techniques were used to recover the HBV volatile fraction. The first one was performed by analyzing the volatile compounds present in the whole sting worker-bees apparatus extract (198,238). The second takes advantage of a solid-phase microextraction (SPME) fiber to collect alarm pheromones and volatiles from the airspace around bees captured in vials (69). The last one represents a new and astute technique. It is a dry air pulsating device at a constant temperature of 30°C. The created airflow carries away bee venom volatile components after exiting bees to sting and pierce a porous membrane. Subsequently, by condensation at a temperature of -90°C, the volatile constituents were brought into solution; afterward, the analysis will be carried out with gas chromatography-mass spectrometry (GC-MS) (21). This technique yielded quantitative and qualitative differences in volatile substances either in stings or in fresh bee venom. This variation is related to the denaturation process, which is immediately triggered after the bee venom's extrusion next stinging or extraction. 3% of bee venom volatiles were essential oils, mainly esters, and isoamyl acetate is the main volatile component of fresh bee venom (21,198).

3.4 Metals and mineral identification:

Metals' content monitoring of process intermediates and final drug substances, including natural products like HBV, is crucial. Nonetheless, there is not enough information about metal contamination in honey bee venom in the literature (74). Consequently, since there are no reference materials with certified metal contents in honey HBV, two relevant reference materials with similar metal levels in bee venom can be used. SLRS-4 (River Water Reference Material for Trace Metals) and ERM-CA021a (Soft Drinking Water). Inductively coupled plasma mass spectrometry (ICP-MS) has been validated for analyzing the contamination of 20 elements. According to the statistical analysis from this method, there is a year to year significant differences in the content of Cu, Na, Ca, Al, V, Mn, Co, As, Sr, Mo, Ba, and Pb. The analysis showed that the content of toxic metals (As, Ba, Pb, Cd, Sb, and Cr) in investigated samples was much lower than the permissible levels for drug substances (74).

3.5 Trace substances:

3.5.1 Sugar:

There is an overwhelming lack in the literature of reports referring to analytical techniques of free sugar characterization in honey bee venom. The rarity, the structure similarities, and the hydrophilic behavior of underivatized sugars, likewise the low proton affinity and ionizability of oligosaccharides, make their characterization tricky (55). However, several studies have been performed to identify protein-linked sugars. *N*-linked oligosaccharides of HBV PLA2 and hyaluronidase were the most investigated (54,239–241). μHPLC-ESI-MS and fluorophore-assisted carbohydrate electrophoresis (FCHE) can be used to detect N-linked oligosaccharides (55,242). LC-Q-Orbitrap-MS untargeted metabolomics analysis of HBV was used to identify and quantify three hexoses, including Glucose, fructose, and vanilloloside (193).

3.5.2 Free amino acids:

Amino acids constitute the essential precursors of HBV catecholamines, peptides, and protein biosynthesis (243). Many methods were used for the separation, identification, and quantification of free amino acids for HBV. Gel filtration, paper- and thin-layer chromatography were used to estimate the part of HBV-free amino acids (less than 1%) and to quantify nineteen HBV-free amino acids, which arginine, cystine, glutamic acid, and histidine are in the majority (202). RP-HPLC and electrochemical detection allowed the identification of eight HBV-free amino acids with amount differences depending on the species, including aspartate, glutamate, serine, alanine, glycine, phenyl-ethanolamine, γ-aminobutyric acid, and tyrosine (243). LC-Q-Orbitrap-MS untargeted metabolomics analysis of HBV showed that amino acids and derivatives were the most common metabolite class found in HBV (nearly 40% of all metabolites in HBV). To quantify a broad spectrum of amino acids, two targeted metabolomics experiments were achieved. The Wide-spectrum metabolite determination using Absolute IDQ p180 kit and LC-QqQ-MS/MS allowed determining 40 amino acids, including glycine, alanine, lysine, taurine, arginine, glutamic acid, and aspartic acid. However, the Wide-spectrum amino acid determination using aTRAQ kit and LC-QqQ-MS/MS enabled to identify 42 amino acids, including proteogenic amino acids (proline, serine, leucine Glutamine, glutamic acid, etc.) and non-proteinogenic amino acids (taurine, γ.aminobutyric acid, ethanolamine, β-alanine, etc.) (193).

Chapter III: Methodology.

4 Bee venom extraction and sample preparation

4.1 Improvement of a device for bee venom collection:

Collecting bee venom or apitoxin requires careful work with the highest degree of cleanliness. Furthermore, precautions at the time of collection in the apiary must be considered. Several kinds of bee venom collectors exist in the market of beekeeping equipment. Apparatus used outside the hive, the collector's panel is put on the entrance or the hive's bottom board. Other types are used inside the hive, on or instead of the frames. This improvement is an essay to provide some enhancements to a bee venom collector prototype obtained from Romania. We focused on several key points, including the quality of HBV, namely the physicochemical characteristics and the purity of the product, the HBV yield, the colonies' stability and the apiary, the decrease of bee damages, and finally, an affordable device for Moroccan beekeepers. The improvement affects several levels and sections, including the electronic control system part, stainless wire array, and venom collection surface.

The implementation of our device relies on various stages:

Finding out the best current (Figure 18): commonly, the most widespread method of bee venom collection is by stimulating bees with electric impulses. A sinusoidal current is tested. With the diode, the negative half of the alternating current is blocked, so only the positive half of the input waveform reaches the output. Consequently, a unidirectional but pulsating direct current is yielded.

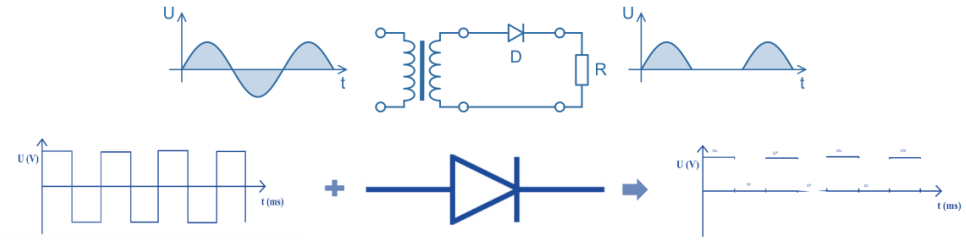


Figure 18. Diagram showing the transformation of sinusoidal current to half-sinusoidal current.

An enhancement design of the collector's frame (Figure 19): on the one hand, the principal concerns were to design a bee venom frame that allows high-quality bee venom collection and the best output with respecting the strength of beehives, and on the other hand, building a device that at least provides a valuable alternative to existing bee venom collectors.

The apparatus comprises a frame supporting the venom collection glass and suspending the wire array on both sides of the plate and a tensioner to hold the wire array tighter. Each frame has only one venom collection glass, but the two sides are exploitable. The collector is used inside the hive and can be put anywhere in it, as shown in (Figure 19). The bee venom collection glass is covered with a specific plastic, generally without harm to bees' stingers.

The optimum electrostimulation parameters are as follows: A half sinusoidal current powers the device extraction, the Impulse frequency at 25 Hz, the impulse voltage increases from 0 V to 12 V then decreases to 0 V, the impulse duration: is 1 ms, and the interval between impulses: 1 ms. Both values, impulse duration, and the gap between impulses are deducted from the source and given by the converter. The conducted experiments showed that the best method consists of placing the frame at one of the beehive's outermost opposite ends. An average yield per essay (30 to 60 min) and by beehive was from 30 to 35 mg of dry bee venom. The quality was promising, as we can observe in Figure 20.

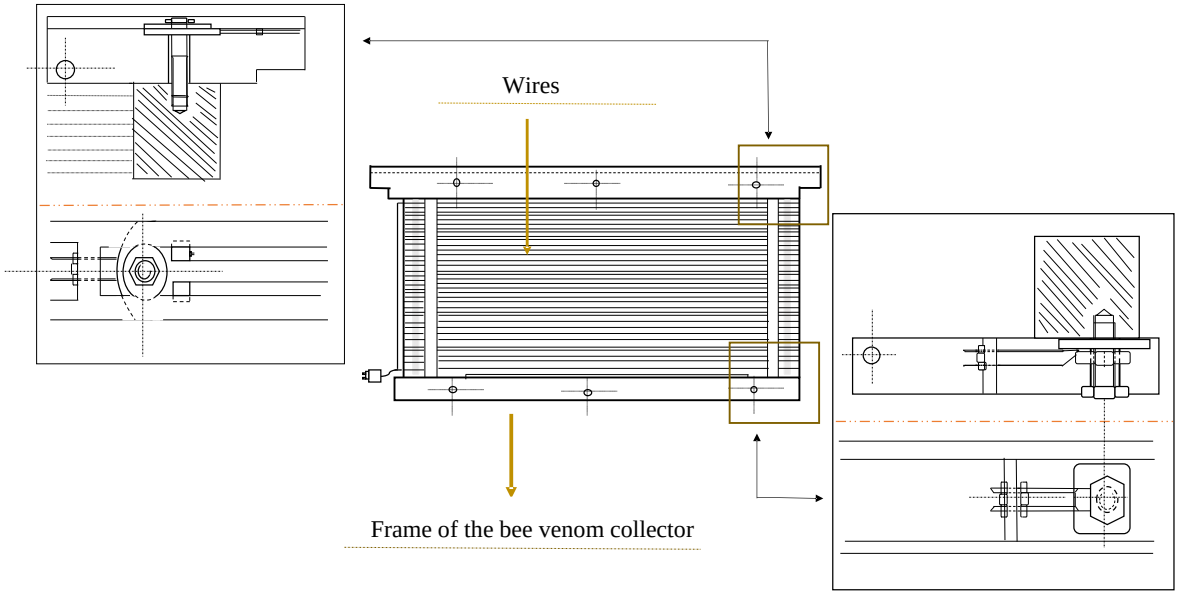


Figure 19. The developed double-face bee venom collector with a schematic diagram of the equipment

Figure 20. Collected HBV by our device (in the center) compared to the one from the beekeeping market (on the right and left).

Be venom is dried in clean conditions with minimum germs and without secondary air current with the present apparatus. We have fewer died and injured worker bees (15 to 30 bees/device/period; data not shown). The presence of a wire tensioner provides excellent current conductibility, and thus an optimum bee electrocuting.

This device made in our laboratory will contribute to providing the raw material locally for subsequent studies and allow expanding beekeeping activities, including honey, bee wax, propolis, and bee bread harvesting. Thus, additional value to beekeeper's incomes.

4.2 Sample preparation and conservation:

Fifteen HBV samples were collected from three regions of Morocco, five samples from each region (northeast, 34°59'48.0"N 2°23'35.7"W; center, 32°32'05.7"N 6°22'31.5"W, and southern Morocco, 30°37'37.3"N 5°27'17.6"W) from August to November 2018 in addition to one sample from August 2017 from the same site of northeast Morocco. The samples were collected by a double-face bee venom collector developed in our laboratory with some exertional features and subjected to a patent. The device was placed in the hive at one of the outmost opposite ends of the beehive. A mild electrical impulse shock (increases from 0 v to 12 v then decreases to 0 v) was applied. The optimum frequency of venom-collecting events is 10 to 15 days for each colony. The optimum duration of bee venom collection in the beehive is 30 min to 60 min early in the morning or at the beginning of the sunset. After the collection session, the venom was scraped off from the glass with a sharp scraper and conditioned in conforming vials.

The conditioning and the storage should respect some precautions, including dark vials at a pharmaceutical-grade, storage-temperature set at least 20°C, and a transfer in the darkness at the same temperature and on the same day.

5 Chemical and biological characterization of HBV samples:

5.1 Standards and reagents:

Apamin (purity 98.3%) was obtained from CalBiochem (San Diego, CA, USA). Melittin (purity ≥ 85%, HPLC), PLA2 from bee venom (activity: 1775 units mg^{-1} solid), Cytochrome *c* from the equine heart (purity ≥ 95%), lipopolysaccharide (LPS), ellipticine, dexamethasone (DM), sulforhodamine B, trypan blue, trichloroacetic acid (TCA), and Tris were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Formic acid was from Panreac (Barcelona, Spain), while HPLC grade acetonitrile was from Fisher Scientific UK (Loughborough, Leics, UK). Standards for metal analysis were purchased from PanReac (Barcelona, Spain). RAW 264.7 cells were from ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK) and DMEM from Hyclone (Logan, Utah, US). The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA). Dulbecco's Modified Eagle's medium (DMEM), hank's balanced salt solution (HBSS), Foetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Gibco Invitrogen Life Technology (California, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

5.2 Chemical characterization of HBV samples:

5.2.1 LC-ESI/MS^n:

The LC-DAD-ESI/MS^n analyses were performed on a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, USA) equipped with a diode-array detector and coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an autosampler maintained at 5 ºC, a degasser, a photodiode-array detector, and an automatic thermostatic column

compartment. The chromatographic separation was carried out according to previously described work with some modifications on an XSelect CSH130 C18, 100 mm × 2.1 mm id, 2,5 μm XP column (Waters, Milford, MA), and its temperature was maintained at 30 °C. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile, which was previously degassed and filtered. The solvent gradient started with 100% A, reaching 60% B at 12 min, 100% B at 15 min, and returned to the initial conditions. The flow rate was 0.3 mL/min, with an injection volume of 40 μL. Spectral data of all peaks were accumulated in the range of 190-500 nm. Cytochrome *c*, as the internal standard (IS), was prepared in deionized water at a concentration of 25 μg/mL. The lyophilized bee venom (3 g) was dissolved in 10 mL of IS for the analysis. Each sample was filtered through a 0.2 μm nylon membrane (Whatman). The analytical parameters of the calibration curve for apamin, PLA2, and melittin are given in (Table 8). The standard solutions were prepared by dissolving them in IS solution for the desire Concentration. The mass spectrometer was operated in the positive ion mode using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an ESI source. Typical ESI conditions were nitrogen sheath gas 35 psi, spray voltage 3,5 kV, source temperature 300 °C, capillary voltage 20 V, and the tube lens offset kept at a voltage of 74 V. the collision energy used was 30 (arbitrary units). Data acquisition was carried out with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA) (Figure 21).

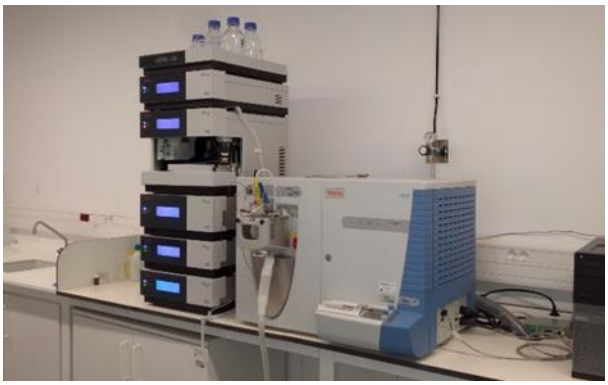


Figure 21. LC-DAD-ESI/MS^n. Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, USA) coupled to Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA)

Table 8. Analytical parameters of the calibration curves of apamin, PLA2, and melittin (n = 5).

	Calibration curve type	Linear range (µg/mL)	Slope	Intercept	Regression coefficient (R^2)
Apamin	1	3.75-60	0.0306	0.0207	0.9985
	2	-	-	-	-
PLA2	1	7.5-120	0.027	0.2511	0.977
	2	7.5-120	0.0263	0.1181	0.996
Melittin	1	31.25-500	0.0346	0.1047	0.9984
	2	31.25-500	0.1047	0.0285	0.9986

5.2.2 Near-infrared analysis of the samples (NIR):

The samples' NIR spectra were obtained according to the methodology proposed by (244), using a BRUKER spectrometer operated with the OPUS®, version: 7.5.18 (Bruker Optik, Germany) software in reflectance mode (Figure 22). About 10 mg of bee venom was poured into a clean glass vial with a 22 mm diameter. The samples were measured with a spectral resolution of 8 cm^{-1} and 32 scans in the wavenumber range between 4000 and 10000 cm−1. About 10 mg of HBV was poured into a clean glass vial with a 22 mm diameter.

Figure 22. BRUKER spectrometer operated with the OPUS®, version 7.5.18 (Bruker Optic, Germany) software in reflectance mode.

5.2.3 Metal determination in samples by atomic absorption spectroscopy (AAS):

The sample analyses were performed in a Perkin Elmer PinAAcle 900T model atomic spectrometer with a flame and a furnace (Figure 23). The flame was used for assessing potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), copper (Cu), and zinc (Zn), and the experimental conditions are shown in Table 9. The analysis of cadmium (Cd), manganese (Mn), nickel (Ni), and lead (Pb) was carried out in a furnace with Zeeman for background correction. The temperature program is described in Table 10.

Table 9. AAS operating conditions and measurement parameters for K, Na, Ca, Mg, Cu, and Zn.

	K	Na	Ca	Mg	Cu	Zn
Flame			Acetylene - Air			
Wavelength (nm)	766.49	589.00	422.67	285.21	342.75	213.86
Slit (nm)	0.2	0.7	0.7	0.7	0.7	0.7

Figure 23. Perkin Elmer PinAAcle 900T model atomic spectrometer

Table 10. Temperature program of Cd, Cr, Mn, Ni, and Pb analysis by GF-AAS

	Drying stage 1			Drying stage 2			Drying stage 3			Pyrolysis			Atomization
	T/°C	t/s	hold	T/°C	t/s	hold	T/°C	t/s	hold	T/°C	t/s	hold	T/°C
Cd	110	1	30	130	15	30	150	15	20	500	10	20	1500
Cr	110	1	30	130	15	30	150	15	20	1500	10	20	2300
Mn	110	1	30	130	15	30	150	15	20	1300	10	20	1900
Ni	110	1	30	130	15	30	150	15	20	1100	10	20	2300
Pb	110	1	30	130	15	30	150	15	20	850	10	20	1600

5.3 Biological characterization of HBV samples:

5.3.1 Anti-inflammatory properties:

According to a method described previously, the anti-inflammatory properties were evaluated using a murine mouse macrophage (RAW 264.7) cell line (129). Cells were routinely maintained as adherent cell cultures in DMEM medium containing 10% FBS, 2mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and kept at 37°C in a humidified air incubator containing 5% CO_2. The BV samples were dissolved in water at 4 mg/mL and then submitted to further dilutions from 4 to 0.0625 mg/mL. RAW264.7 cells (1×10^5 cells/mL) were preincubated for one hour with various HBV concentrations and

stimulated with LPS (1 μg/mL) at 37 °C for 18 h in the medium. According to the authors, nitric oxide (NO) levels were determined by measuring nitrite levels in the culture media using Griess Reagent assay (129). The results were expressed in percentages of inhibition of NO production in comparison with the negative control (without LPS), and IC50 values, equal to the sample concentration providing 50% of inhibition of NO production were also estimated. Dexamethasone was used as a positive control.

5.3.2 Cytotoxic properties:

The HBV samples were dissolved in water at 4 mg/mL and then submitted to further dilutions from 4 to 0.0625 mg/mL. The cytotoxic effects were evaluated using five human cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (Hepatocellular carcinoma), obtained from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), and MM127 (human malignant melanoma) from the ECACC General Cell Collection. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and kept at 37 °C in a humidified air incubator containing 5% CO_2. According to the procedure described previously, HBV's cytotoxic potential was evaluated by the sulforhodamine B assay (245,246). For the hepatotoxicity evaluation, cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to an established procedure, and labeled as PLP2 (porcine liver primary culture) (247). The cell cultivation was maintained, with direct monitoring, every two to three days using a phase-contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Ellipticine was used as a positive control, and the

results were expressed in GI_{50} values (Concentration that inhibited 50% of the net cell growth). Ellipticine was used as a positive control.

5.4 Data analysis:

All experiments were performed in duplicate. Results are expressed as the Mean ± SD. One-way analysis of variance (ANOVA) was performed. Tukey HSD Post Hoc test was performed to look for whether there were significant differences between regions. Afterward, the Welch test was applied as a robust test of equality of means. Pearson's correlation analysis was a tool to establish possible correlation links between HBV chemical composition and its biological activities and to assess whether there are any influences of the factor (region). Statistical analyses were conducted using computer-based statistical software (IBM, SPSS v. 20.0); a *p*-value ≤ .05 was considered statistically significant. A principal component analysis (PCA) was performed for NIR spectra analysis using the Unscrambler® X version: 10.5.46461.632 (CAMO Software AS, Oslo, Norway).

Chapter IV: Results and discussion.

6 LC/DAD/ESI-MS^n analysis:

Knowledge about the composition of HBV recently has been increased by the progressively rapid improvement of analytical techniques. LC/MS methods are becoming widely more common in venomics, especially in chemical HBV characterization (26,129,175,210,213). In this work, we employed LC-ESI/MS^n in the positive ion mode because it is easier to characterize the main components of HBV, including apamin, PLA2, and melittin (26). Full scans were performed over a range *m/z* of 50-2000. Results were reported in (Table 11). The chromatographic profile of all samples from the three regions displayed similar patterns. Thence, we presented only a sample chromatogram from each area in addition to sample NE5a (about a year older than the others). The differences could be seen either in the height of some peaks or at the different samples' Rt occurrences. 4.7 min, 8.5 min, and 9.7 were *Rt* for apamin, PLA2, and melittin, respectively. These chromatograms showed that melittin represents the most significant amount, followed by PLA2 and then apamin (Figure 24).

Analogous results were obtained with those in prior studies with some specifications (Table 12). the HBV showed low values of PLA2, almost less than half. As claimed in the foregoing reports, these differences were related to species, climate, and geographic abundance. Therefore, Ionete et al. showed the greatest values of Apamin and PLA2 (4.09%, and 15.13%, respectively), while Sobral and coworkers made the high value of melittin. (129,175,187,191,215). Statistical analyses displayed homogeneity of variances for apamin and PLA2, but this feature is violated for melittin. Overall, as depicted in (Figure 25), no significant differences concerning apamin and melittin between regions; $p > .05$ (Table 16). Two samples from the southern region recorded High values (S3) and small values (S5) of the amount for apamin and melittin (1.45 ± 0.02; 2.55

± 0.03) and (62.34 ± 0.84; 87.70 ± 0.58), respectively. While for PLA2, significant differences were shown between northeastern and southern regions $F(2,12) = 4.02$, $p = .04$.

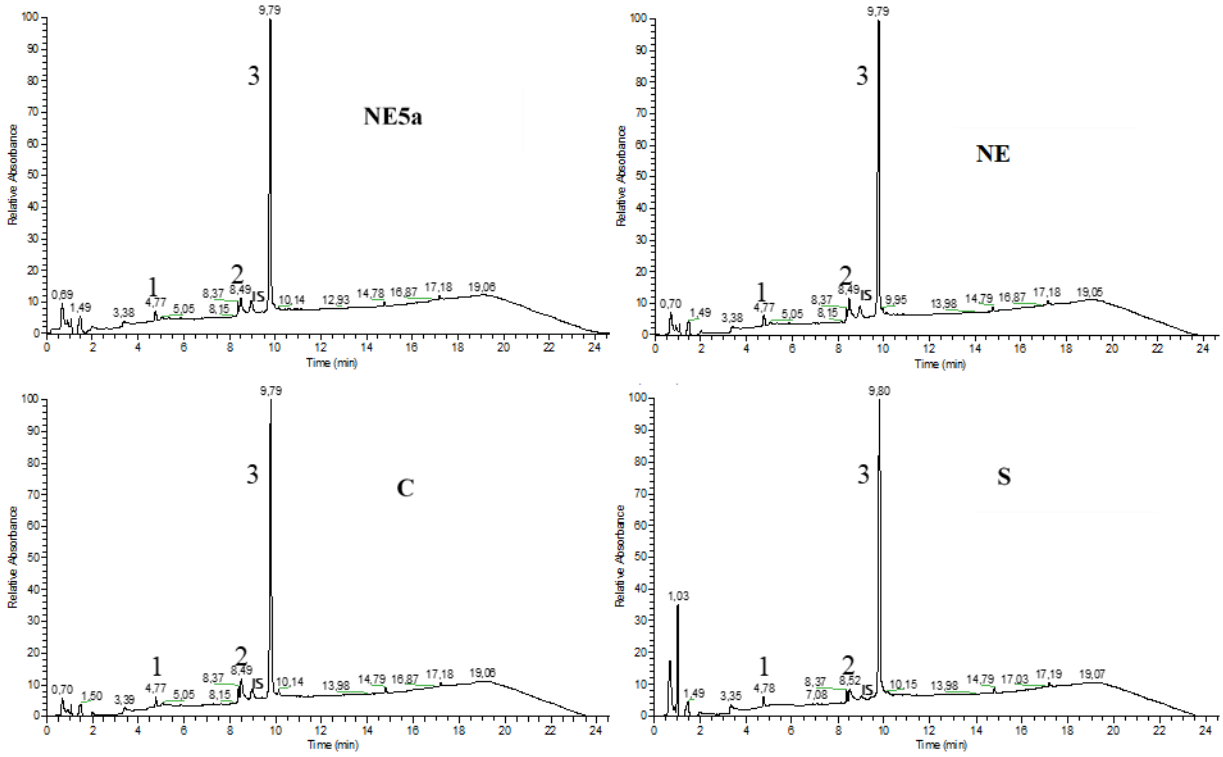


Figure 24. Representative Chromatographic profiles at 220 nm of *Api mellifera intermissa* HBV(one from each region + NE5a, older than one year). NE, Northeastern; C, Center, and S, Southern Morocco. 1- apamin, 2- PLA2, 3- melittin, and IS- internal standard (cytochrome c, 25 μg/mL).

We recorded the highest value in the NE1 sample (9.13 ± 0.13) and the lowest in the S2 sample (4.18 ± 0.03). Sample NE5a (older than one year to others but conserved at – 20 °C) preserved all chemical features except a slight decrease of melittin amount (65.97 ± 0.77) in comparison with the averages within the NE region and between the three regions (NE,71.71 %; C, 71.06 %; S, 75.66 %).

The full scan mass spectrum of apamin, melittin, and PLA2, in addition to the MS2 fragmentation of the precursor ion of apamin and melittin, was presented in Figure 26. The spectra shown were similar to those reported by Zhou et al.,

2010 and Frangieh et al., 2019. The molecular ions with *m/z* 407.08, *m/z* 508.19, and *m/z* 677.10 corresponded to apamin.

Table 11. Chemical characterization of *Apis mellifera intermissa* HBV from northeastern, central, and southern Morocco by LC-DAD-ESI/MSn

	Apamin (µg/mL)	PLA2 (µg/mL)	Melittin (µg/mL)
NE1	2.08 ± 0.01	9.13 ± 0.13	67.16 ± 0.18
NE2	1.93 ± 0.03	6.66 ± 0.37	73.43 ± 0.19
NE3	2.29 ± 0.02	6.57 ± 0.39	78.14 ± 0.30
NE4	1.99 ± 0.04	7.40 ± 0.28	73.85 ± 0.9
NE5a	1.96 ± 0.01	8.54 ± 0.75	65.97 ± 0.77
C1	1.86 ± 0.01	4.21 ± 0.08	71.78 ± 0.14
C2	2.05 ± 0.03	5.05 ± 0.09	73.66 ± 0.92
C3	1.96 ± 0.01	7.14 ± 0.13	71.98 ± 0.48
C4	2.10 ± 0.03	8.03 ± 0.51	75.10 ± 0.29
C5	1.99 ± 0.02	6.42 ± 0.25	70.15 ± 0.36
S1	1.53 ± 0.10	4.52 ± 0.02	66.78 ± 3.43
S2	1.69 ± 0.03	4.18 ± 0.03	71.29 ± 1.24
S3	2.55 ± 0.03	7.81 ± 0.17	87.70 ± 0.58
S4	1.79 ± 0.01	1.79 ± 0.01	70.29 ± 0.87
S5	1.45 ± 0.02	4.45 ± 0.01	62.34 ± 0.84

Table 12. Mean content (%) of the main honey bee venom component (apamin, PLA2, and melittin) compared with previous studies.

	Present Study		(129)	(191)	(210)	(175)
Apamin	NE	2.05	2	4.09	2.64	3.46
	C	1.99				
	S	1.80				
PLA2	NE	7.66	13	15.13	13.04	12.73
	C	6.17				
	S	5.15				
Melittin	NE	71.71	86	58.45	54.08	64.61
	C	72.54				
	S	75.66				

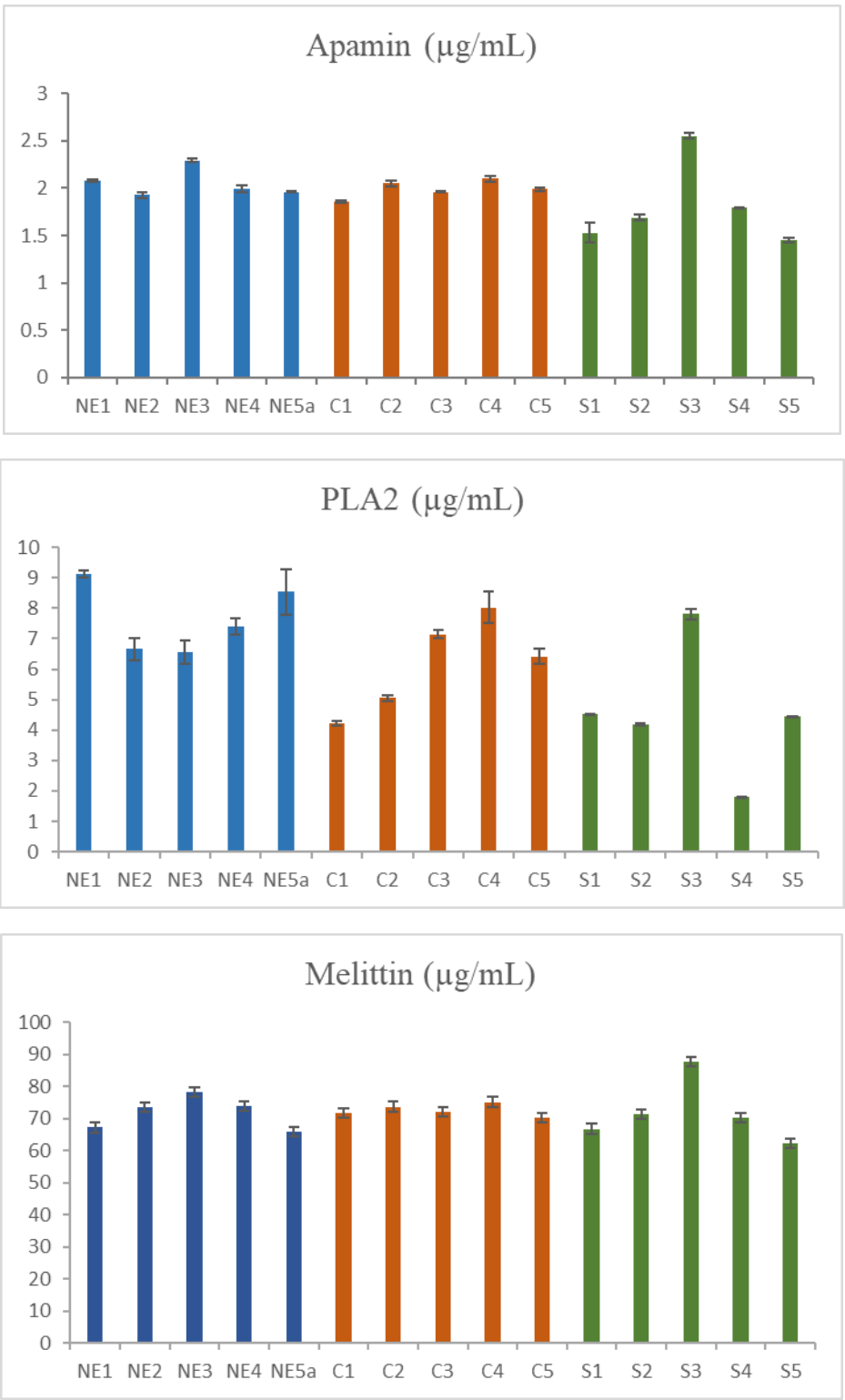


Figure 25. amounts of apamin, PLA2, and melittin by region. NE, Northeastern; C, Center; S, southern Morocco.

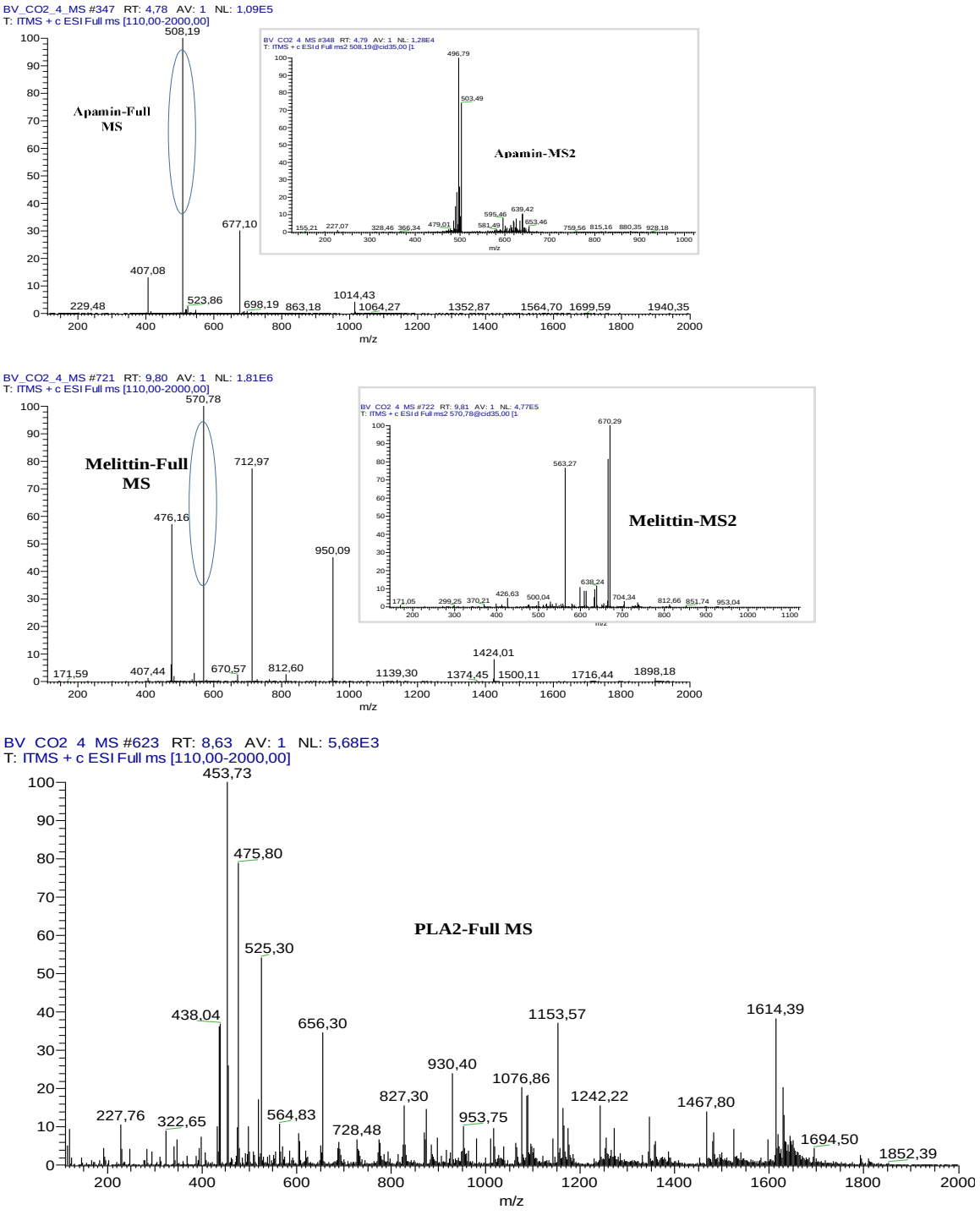


Figure 26. Full scan mass spectrum of apamin, PLA2, melittin, and the MS2 spectrum of the most abundant region with m/z of 508.19 and m/z of 570.78 for apamin and melittin, respectively.

While the molecular ions with *m/z* 476.16, *m/z* 570.78, *m/z* 712.97, and *m/z* 950.09 were specific for melittin. Molecular ions with *m/z* 508.19 *and m/z* 570.78 were submitted to a second mass analysis (MS2) as precursor ions for apamin and melittin. In this MS2 stage, the mass product ions with characteristic fragments were selected and detected. Two product ions for each precursor ion with *m/z* 496.79, *m/z* 503.49 for melittin and *m/z* 496.79, and *m/z* 503.49 for apamin. Concerning PLA2, overall, we can catch from its full scan mass spectrum that the molecular ion is situated at *m/z* 453.73.

7 Metal determination by AAS spectroscopy:

Unfortunately, scarce information can be found in the literature about metal determination in HBV, mainly the most toxics and classified in the European Medicines Agency (EMEA) and the United States Pharmacopeia (USP), including lead (Pb), mercury (Hg), bismuth (Bi), arsenic (As), antimony (Sb), tin (Sn), cadmium (Cd), silver (Ag), copper (Cu), molybdenum (Mo), vanadium (V), Palladium (Pd), platinum (Pt), gold (Au), and ruthenium (Ru) (74,248). Since we are in the process of establishing a quality control procedure for honey bee venom as a raw material for pharmaceutical use, the assessment of elemental impurities needs to be submitted to quality control at the pharmaceutical level. In this framework, we adopted two essential reference materials, the EMA/ICH Q3D guideline for elemental impurities, and the USA (231) and Ph. Eur. (2.4.8) chapters on the determination of heavy metals (249,250). This survey section (Annex. D) reports AAS analyses of the HBV samples collected from the three regions, northeastern, central, and southern Morocco (Table 12). There are two categories of potential sources of elemental impurities, those arising from the bee's environment, including soil natures, fertilizers, and pesticides use, nearby mines, especially coal mines; case of northeastern Morocco, and those related to the process of honey bee venom collection, including combustible material

nature used for the smoke generation, glace plates and scraper nature, and closure and container system used for the product conservation (74). Overall, calcium is the major microelement present in our HBV samples with a concentration around 3000 ppm, followed by potassium, sodium, zinc, and magnesium. All these elemental impurities (with low inherent toxicity) in addition to lead (human toxicant) presented significant differences between regions, $p \leq .05$ (Table 15).

Table 13. Summary table of metal concentration in *Apis mellifera intermissa* HBV samples from the three regions of Morocco

Samples	K (mg/g)	Na (mg/g)	Ca (mg/g)	Mg (mg/g)	Zn (mg/g)	Cu (µg/g)	Cd (µg/g)	Cr (µg/g)	Mn (µg/g)	Ni (µg/g)	Pb (µg/g)
NE1	2.97	1.30	3.25	0.52	1.23	5.37	<5.4ppb	5.20	2.02	26.98	6.13
NE2	3.01	1.34	1.78	0.44	1.39	24.72	<5.4ppb	9.46	4.46	4.32	5.15
NE3	2.64	1.53	1.80	0.33	1.05	4.36	<5.4ppb	7.61	1.61	1.36	9.85
NE4	2.16	1.00	0.95	0.32	1.04	<0,02ppm	2.26	3.86	1.10	<1.6ppb	8.08
NE5	3.57	1.55	1.76	0.57	1.03	4.13	<5.4ppb	8.63	2.79	0.49	6.77
C1	1.62	1.93	4.55	0.42	1.03	9.45	<5.4ppb	4.59	2.47	2.83	3.81
C2	1.73	1.01	2.16	0.27	1.12	<0,02ppm	<5.4ppb	3.41	1.59	0.64	3.56
C3	3.02	1.84	3.22	0.55	1.42	20.91	<5.4ppb	6.71	3.10	0.62	3.99
C4	3.03	1.43	2.32	0.56	1.27	6.83	<5.4ppb	4.99	2.07	4.49	4.84
C5	2.50	0.98	2.59	0.38	1.53	7.30	<5.4ppb	8.29	3.76	1.78	5.16
S1	1.55	0.79	0.96	0.28	0.93	<0,02ppm	<5.4ppb	2.40	0.78	<1,6ppb	7.60
S2	1.68	0.81	0.97	0.27	1.03	3.95	<5.4ppb	2.45	1.19	<1,6ppb	3.63
S3	1.95	0.81	0.83	0.31	0.95	4.10	1.32	13.21	0.89	<1,6ppb	3.24
S4	1.72	0.92	1.41	0.25	1.07	<0,02ppm	<5,4ppb	5.19	1.29	<1,6ppb	4.32
S5	1.56	0.79	0.95	0.25	1.07	3.95	<5,4ppb	5.55	2.19	<1,6ppb	4.20

The southern region showed the lowest mean values while the central region displayed the highest mean concentrations for Na, Mg, Ca, and Zn. As for K, and northeastern region recorded the largest Pb mean values. Concerning the other metals subjected to our investigations, they are present in all samples from the different study sites but without significant differences. However, we noted some observations; Northeastern and center presented the highest mean values of almost all elemental impurities. Heavy metals (Pb, Cr, and Cd) were very concentrated in the samples from the northeastern region but similar to some extent to those made by Kokot et al., 2018 (74). Generally, results were in the range of those reported in the literature; however, sodium and calcium levels were 2 to 3 folds higher. Micronutrients or trace elements (toxic at increased concentrations) Mn, Cu, and Ni were also identified in the samples but were relatively low. Even though some samples showed large concentrations of heavy metals mentioned in the ICH Q3D and the US Pharmacopeia, they are still in the recommended ranges (≤ 5 ppm) indicated by the reference materials mentioned above (Table 13).

According to the obtained data, AAS spectroscopy can be considered a useful tool for HBV standardization and authentication control for pharmaceutical use. However, establishing a good beekeeping practice (GBP) for HBV collection is necessary, including beekeeper's qualification, hygiene measures respect, collecting conform materials, documentation, labeling, and storage.

Table 14. Comparison of the obtained values of metal concentration in HBV samples from the three regions (in mean values) and the oral, parenteral, and inhalation permeated concentration in µg/g for elemental impurities in the drug product, drug substances, and excipients set by the ICH guideline Q3D on elemental impurities (251).

	Class	Oral Concentration	Parenteral concentration	Inhalation concentration	HBV concentration	
Cd	1	0.5	0.2	0.2	NE	0.456
					C	0.005
					S	0.26
Pb	1	0.5	0.5	0.5	NE	7.196*
					C	4.272*
					S	4.592*
Ni	2A	20	2	0.5	NE	6.650
					C	2.072
					S	0.001
Cu	3	300	30	3	NE	1.908
					C	2.968
					S	1.024
Cr	3	1100	110	0.3	NE	6.952
					C	5.598
					S	5.760

8 NIR spectroscopy:

NIR spectroscopy is a common analytical technique used to identify and quantify the chemical composition of different products in foods, pharmaceuticals, the petroleum industry, and other fields. Its versatility, non-destructive, non-invasive, fast, and precise method made it a useful analytical technique (252–254). In this investigation part, we used infrared spectroscopy to explore possible differences between the fifteen samples of HBV collected in the three regions under study (Annex. C and E). According to the NIR spectra, there is noticeable information in the region from 9000 to 4500 cm^{-1} (Fig.29). Roughly, the absorption bands for polypeptides (the main compounds of the dry

HBV) appear between 4800 cm^{-1} and 6800 cm^{-1} in the overtone region. The band at 6595 cm^{-1} corresponds to CH_2 and CH_3 linking and the first overtone of the N-H linking. Bands between 5749 cm^{-1} and 5138 cm^{-1} represented the combination of O-H stretch and O-H deformation and bent the second overtone. The band at 4862 cm-1 is assigned by the N-H's vibration mode of the amide II and amine. In the more relevant spectral region, we performed a PCA analysis and a second derivative pretreatment (Fig.30, A2, and B2).

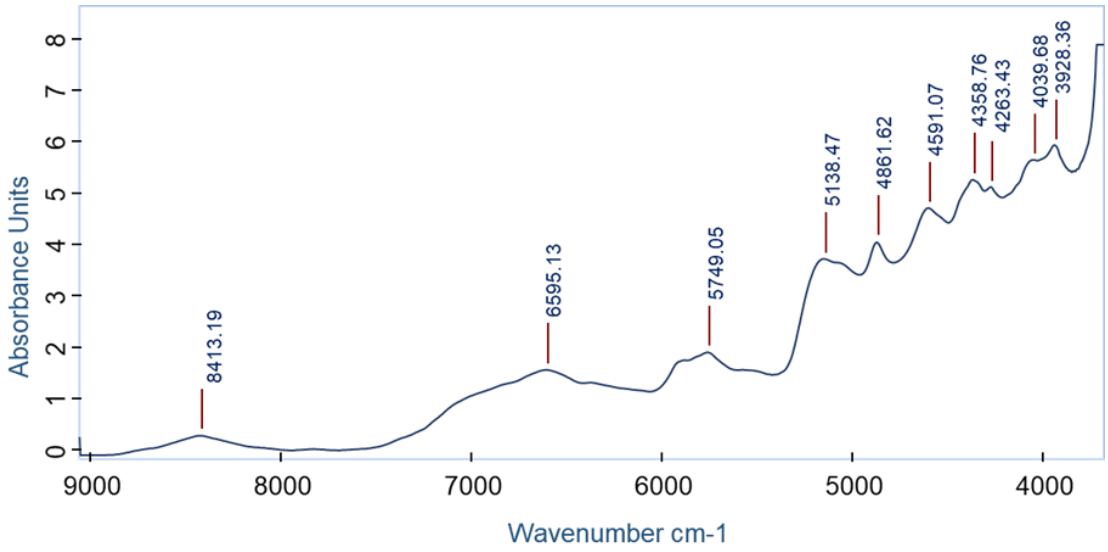


Figure 27. Average HBV NIR spectra with the indication of the more relevant bands

HBV spectra from the same region showed interesting similitudes. However, this product also has low molecular weight compounds, which add relevant spectral information. As exhibited in the PCA score plot B1 (Fig 30), we distinguish three groups (a group for each region). This aspect was corroborated mostly by the chemical composition differences of different samples from the three regions. However, samples NE4, C4, and S4 were in disharmony with the other samples. We can explain this particularity by the significant differences in PLA2 composition between the three regions, especially for these samples, $F(2,12)= 4.2, p = .04$. (Table 15). Additionally, the score plot A1 (Fig 30) demonstrated

the high sensitivity to the samples' storage period, which allowed discrimination among old and fresh bee venom samples.

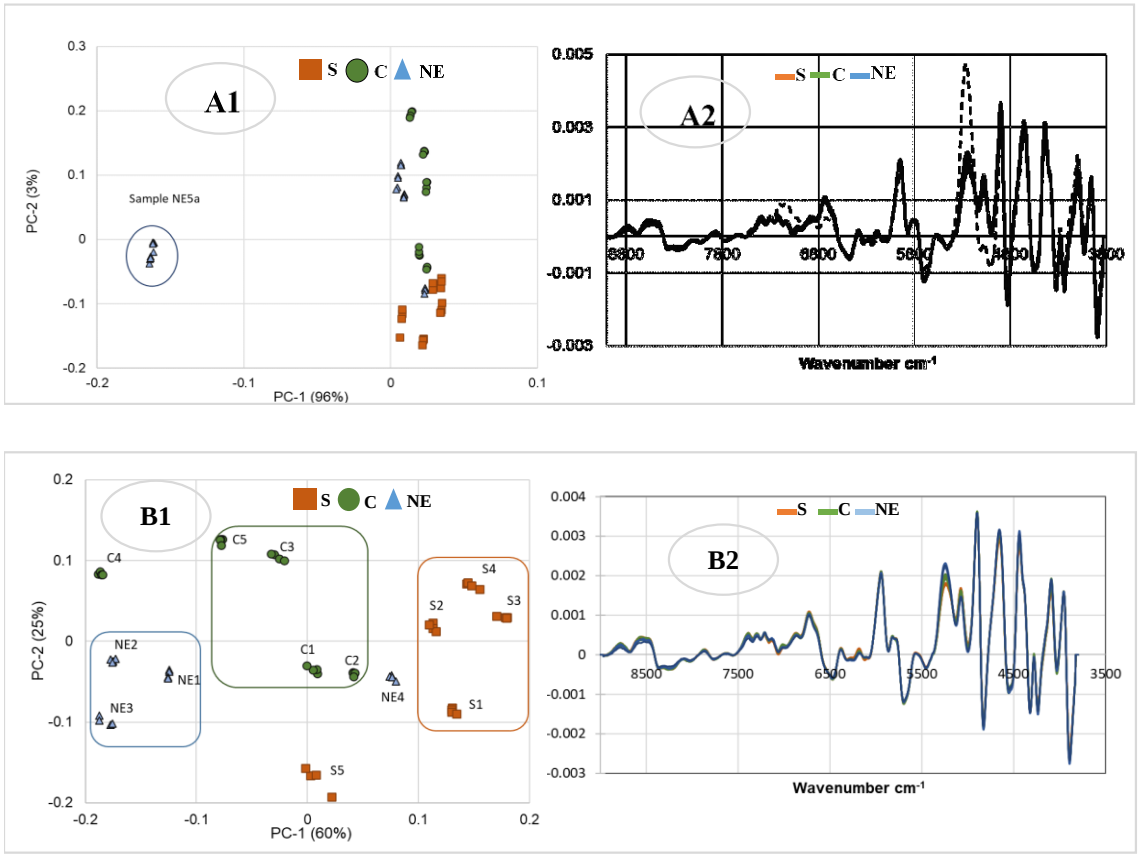


Figure 28. PCA score plot of the more relevant spectral region of NIR spectra of HBV samples from the three regions of Morocco with NE5a (A1) and without NE5a (B1). A2 and B2 displayed the second derivative pretreatment.

9 Cytotoxic and anticancer activity:

Cell-based cytotoxic activity is an earlier stage, among others, to improve the efficacy and safety of new chemical entities in drug discovery (255,256). For *in vitro* cell culture systems, a compound or treatment is considered cytotoxic if it interferes with cellular attachment, significantly alters morphology, and adversely affects cell growth rate or causes cell death (257). Two conventional

assays are usually applied because of their easy operation and standardized readout: electrochemical methods that record impedance related to incubated cells' physiological status on the gold microchips and optical methods that measure cell absorbance viability-sensitive dyes in the tested solution (258).

The cytotoxicity profiles vary depending on compound potency, dosage, and exposure time (257). One of the conventional colorimetric assays (optical method), Sulforhodamine B (SRB), is the preferred high throughput assay of the National Cancer Institute (NCI) in the USA and is the assay used in the NCI's compound screening program. It is the choice method for highly cost-effective screenings (259,260). Thus, we evaluated HBV's cytotoxic activity on five human tumor cell lines, including MCF-7, NCI-H460, HeLa, HepG2, and MM127. We used PLP2 cells to analyze the potential hepatotoxicity since mammalian hepatocytes still represent an obligatory step in evaluating toxic compounds that lead to the production of various metabolites, which are the ultimate cause of toxicity (247). Several reports showed that honey bee venom manifested anticancer activity on a range of animal and human tumor cell lines, including mouse melanoma K1735M2 (171), human hepatoma cell line SMMC-7721(261), human cell leukemic U937 (78), human melanoma A2058 cells (172), malignant melanoma B16F10 cells (262), non-small cell lung cancer cells A459 (263), and human ovarian cancer cell A2780cp (109). In this work, we tested five tumor cell lines. The obtained results are figured in (Table 13) in terms of GI_{50} (μg/mL). All samples from the three regions demonstrated cytotoxic activity for almost all the studied cell lines (Fig 31). Significant differences have been observed between regions for MCF-7 and MM127 and similarities to some extent for HepG2, NCI-H460, and HeLa (Table 15). It seems that HBV samples from the northeastern region had the most potent cytotoxic activity on the different cell lines with the lowest GI_{50}. Sample NE4 well corroborated this trend with the smallest IG_{50}s values on other cancer cell lines (two to five folds more

significant, all cell lines combined) except for sample C3 which presented the best cytotoxic effect on HepG2 cell line with a GI50 = 1.9 μg/mL even outstandingly lower than that obtained with bee venom from north Portugal which GI50 was ranging 5.43 μg/mL to 12.19 μg/mL (129).

Table 15. Cytotoxic and anti-inflammatory activities of HBV from NE, C, and S Morocco (μg/mL)

	Cytotoxic activity (GI_{50}, µg/mL)						AIA* (EC_{50}, µg/mL)
	HepG2	**NCI_H460**	**HeLa**	**MM127**	**MCF7**	**PLP2**	**RAW264,7**
NE1	4,86 ± 0.33	9.57 ± 0.69	4.84 ± 0.35	3.72 ± 0.27	4.64 ± 0.37	13.77 ± 0.51	5,07 ± 0.20
NE2	4.32 ± 0.29	7.35 ± 0.70	3.88 ± 0.16	3.64 ± 0.35	4.20 ± 0.23	15.33 ± 0.37	4.89 ± 0.24
NE3	3.58 ± 0.40	6.88 ± 0.21	3.75 ± 0.42	4.01 ± 0.40	4.17 ± 0.42	27.15 ± 1.79	6.06 ± 0.17
NE4	2.4 ± 0.13	3.09 ±0.15	2.08 ± 0.14	2.05 ± 0.16	2.62 ± 0.08	4.06 ± 0.11	4.03 ± 0.37
NE_{5a}	4.56 ±0 .24	7.77 ±0.51	3.81 ± 0.24	3.80 ± 0.40	4.55 ± 0.45	8.96 ± 0.88	4.86 ± 0.39
C1	4.02 ± 0.37	8.06 ± 0.11	4.15 ± 0.19	4.64 ±0.27	6.40 ± 0.47	15.13 ± 0.26	7.92 ± 0.78
C2	4.10 ± 0.41	8.26 ± 0.10	4.06 ±0.35	4.92 ± 0.29	6.86 ± 0.40	14.84 ± 0.53	7.78 ± 0.59
C3	1.99 ± 0.18	4.12 ± 0.24	2.97 ± 0.28	3.33 ± 0.06	3.91 ± 0.28	13.74 ± 0.50	6.12 ± 0.32
C4	3.94 ± 0.30	7.27 ± 0,25	4.94 ± 0.18	4.44 ± 0.39	5.90 ± 0.16	11.13 ± 0.40	6.26 ± 0.40
C5	3.79 ± 0.06	8.08 ± 0.54	5.36 ± 0.12	4.95 ±0 .19	5.59 ± 0.36	14.14 ± 0.73	6.11 ± 0.47
S1	4.95 ± 0.37	8.85 ± 0.38	4.67 ± 0.44	4.99 ± 0.42	5.94 ± 0.28	18.03 ± 0.24	9.08 ± 0.45
S2	3.98 ± 0.08	7.85 ± 0.40	4.36 ± 0.25	4.49 ± 0.51	5.52 ± 0.54	13.93 ± 0.31	7.40 ± 0.26
S3	4.33 ± 0.45	7.17 ± 0.46	4.67 ± 0.16	4.24 ± 0.08	6.02 ± 0.39	14.86 ± 0.62	6.65 ± 0.34
S4	11.46 ± 0.82	15.32 ± 1.45	8.71 ± 0.45	8.09 ± 0.46	12.18 ± 0.90	38.85 ± 2,83	**15.56 ± 0.81**
S5	15.63 ± 0.66	15.63 ± 0.70	8.35 ± 0.30	7.68 ± 0.34	10.89 ± 0.57	14.12 ± 0.21	15.02 ± 0.34
DM							15.50 ± 1.6

*: Anti-inflammatory activity

In comparison to the previous study, the venom from this species proved an exceptionally cytotoxic activity on NCI-H460, and HepG2 but comparable GI_{50} values on HeLa and MCF-7 (106,129,213,263,264). However, Duffy et al.

showed inconsistent results to ours, for the cytotoxic activity on the MCF-7 cell line with a large GI_{50} of 10.77 ± 0.22 (96).

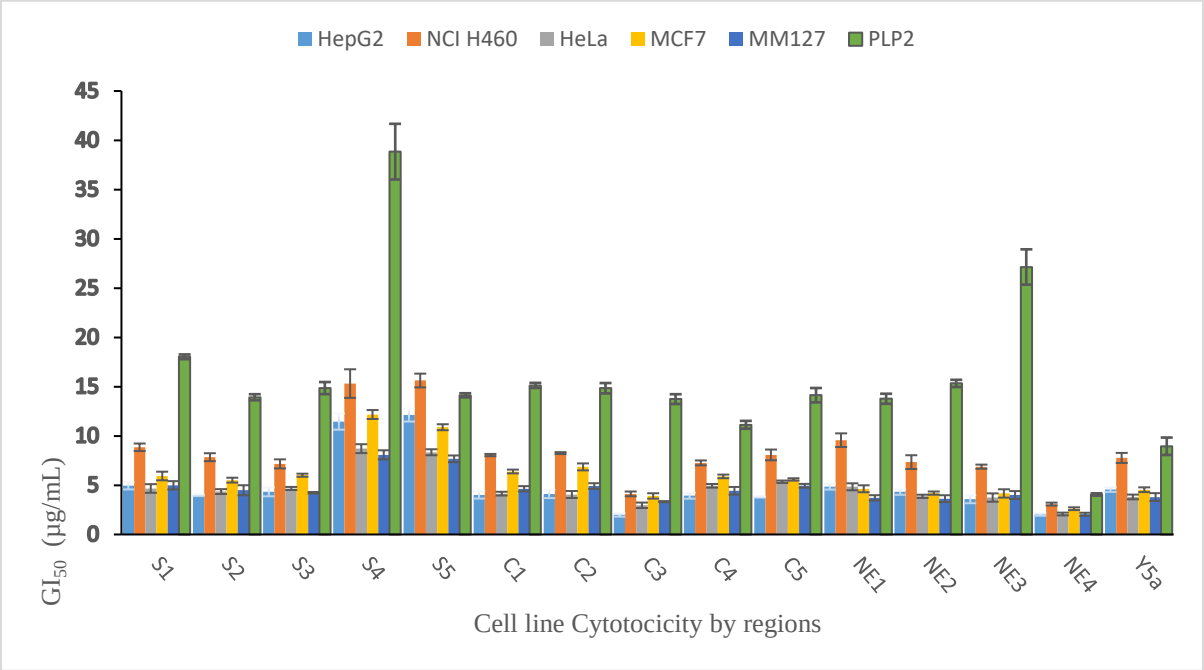


Figure 29. Cytotoxicity activity of HBV samples from the three regions (S, South; W, Center; NE, Northeastern Morocco; NE5a, this sample is a year older than the others). The results are expressed in GI50 values (µg/mL) (Means ± SD, n = 2). GI50 values correspond to the Ami HBV concentration achieving 50% growth inhibition in the five human tumor lines or porcine liver primary culture PLP2.

10 Anti-inflammatory activity:

Honey bee venom from different species has well-proven anti-inflammatory properties *in vivo* and *in vitro* (15,122,125,129,142,265,266). *In* vitro studies were essentially established on pro-inflammatory responses in RAW264.7 cell lines (267). The effective HBV concentration for the inhibition of NO production by LPSs in RAW264.7 cell lines ranged from 0.1 to 20 µg/mL (121,122,268). According to the author, the interval was from 1 to 15 µg/mL (129). Here, in this study, we evaluated HBV's anti-inflammatory potential from different regions of Morocco by assessing the effects of this substrate on the pro-inflammatory responses in the RAW264.7 macrophage cell line. Results are presented in (Table 15) in terms of EC_{50} (µg/mL). Looking at the results

above, we can constate that all samples exhibit anti-inflammatory activity with significant differences between samples from southern and northeastern regions ($p > .05$). While no significant differences between either northeastern and center or center and southern region (Table 15). The best inhibitory activity on NO production was demonstrated by samples from the NE region followed by samples from the center. In contrast, those from the southern region held the lowest activity (Fig.32). It is evident to note that sample NE4 showed a better EC_{50} value than that obtained by *Apis mellifera iberiensis* from Northeast Portugal (129), even more, significant than the result obtained by DM (EC_{50} = 4.03 ± 0.37; EC_{50} = 4.85 ± 0.02; EC_{50} = 15.6 ± 1.6, respectively).

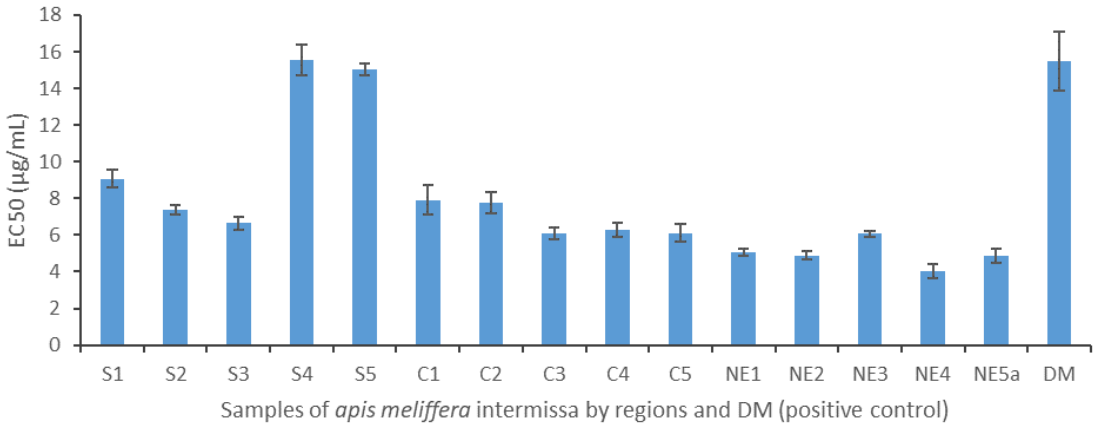


Figure 30. Anti-inflammatory activity of HBV samples from the three regions (S, south; C, center; NE, northeastern Morocco; NE5a, this sample is a year older than the others; DM, dexamethasone). The results are expressed in IC50 values (µg/mL) of NO production inhibition (means ± SD, n = 2). EC50 corresponds to the HBV concentration achieving 50% of the inhibition of the NO production.

Besides, we noted that sample S5a (a year older than other samples) preserved its anti-inflammatory potentiality largely. The sample S4 presented the weakest activity with an EC_{50} superior to that obtained with the positive control (EC_{50} = 15.56 ± 0.81; EC_{50} = 15.6 ± 1.6, respectively).

In summary, fifteen Apis mellifera intermissa venom samples from different regions (northeastern, center, and southern) of Morocco have been submitted for the first time for chemical and bioactive characterization. LC-ESI/MSn

analyses showed that the chemical composition of HBV, mainly the most abundant including melittin, PLA2, and apamin, is notably influenced either by bee environment factors or by collect process conditions. No significant differences in mean amounts were shown between or within regions except for PLA2, which is more concentrated in samples from the center and southern region. This pattern is supported by the data provided by NIR spectroscopy (Fig.30, A1, and B1). Heavy metals and micronutrients or trace elements were found in all samples but kept still in the range recommended by guideline references for pharmaceutical raw materials cited above (< 5ppm). However, results obtained by AAS spectroscopy revealed that there are, albeit less clear, pollution sources that affected lead concentrations in samples from the northeastern region. We might claim and attribute that to the presence of many coal mines in this region. So, an investigation should be done in this context. All HBV samples tested on the six tumor cell lines displayed cytotoxic and anti-inflammatory activities, but only samples from northeastern with the highest concentration mean values in apamin and PLA2 (2.05 ± 0.14 μg/mL, 7.66 ± 1.13 μg/mL, respectively) manifested significant activities on MCF-7, MM127, and RAW264.7 cell lines with the lowest concentrations GI_{50} = 4.03 ± 0.81μg/g, GI_{50} = 3.44 ± 0.79 μg/mL, EC_{50} = 4.98 ± 0.72 μg/mL, respectively (Table.15). Besides, this is consistent to a large extent with the known anti-inflammatory activity essentially for apamin and partially for melittin in addition to synergistic and complementary cytotoxic properties for melittin and PLA2 (82,84,100,269–271). Furthermore, the results of the Spearman/s Rho (Table.16) test showed that there was a moderate negative linear relationship, on the one hand between PLA2 and MM127 melanoma cell lines, and on the other hand between PLA2 and MCF-7 tumor cell lines (r_s (13) = -0.590, p <.05; r_s(13) = -.55, p <.05, respectively). Likewise, between PLA2 and RAW264.7 and between Apamin

and the same cell lines (r_s (13) = -0.652, $p < .01$; r_s(13) = -.560, $p < .01$, respectively).

These findings and prior studies confirmed that HBV and Mlt could be used as a strong candidate in cancer-targeted therapy. It could be used alone or as an adjuvant with currently FDA-approved drugs (272). Their investigation (108) demonstrated that cisplatin, one of the oldest chemotherapeutical drugs used for treating advanced testicular cancers, advanced ovarian cancers, and advanced bladder cancers, can induce apoptosis in cisplatin-resistant human ovarian cancer A2780cp cells. Likewise, the combination of both compounds showed synergistic effects. A2780cp individual treatment by HBV at 8μg/mL and cisplatin at 25mg/mL, caused nearly 50% A2780cp cell death, while treatment with HBV and cisplatin gave a similar A2780cp cell death but with a low concentration of 4μg/mL and 10 mg/mL for HBV and cisplatin, respectively. (110) studied another aspect of the ovarian cancer cells co-treatment with cisplatin and Mlt, but not HBV. They revealed that the combination treatment induced significant changes in A2780cp and A2780CR cells involving a reduction in the levels of metabolites in the tricarboxylic acid cycle, oxidative phosphorylation, purine, and pyrimidine metabolism, and the arginine/proline pathways. This combination has a synergistic effect more significant on A2780cp than A2780CR.

On the same type of human cancer cells A2780cp, (109) showed that HBV and chrysin's co-exposure treatment, a natural flavonoid derived from honey and propolis, was effective for destroying chemoresistant ovarian cancer cells A2780cp. Moreover, a synergistic effect was displayed. The IC_{50} values for A2780cp cells were considered to be approximately 8μg/mL HBV, 40μg/mL chrysin, and 6+15 μg/mL HBV + chrysin.

In contrast, (273) studied the cytotoxic and genotoxic effects of HBV and the chemotherapeutic agent bleomycin (BLM) on healthy isolated rat lymphocytes.

BLM is a water-soluble antibiotic with anticancer activity (274). They found that HBV potentiates BLM-induced cytotoxicity through increased lactate dehydrogenase release and diminished cell viability. However, HBV significantly attenuated the genotoxic effects of BLM on noncancerous isolated rat lymphocytes.

Furthermore, (88) proclaimed that HBV synergistically enhances the anticancer effect of tamoxifen, a chemotherapeutical drug widely used in HER-positive breast cancer treatment, and could be used as an adjuvant/vehicle to tamoxifen in breast cancer treatment.

Mlt synergistically potentiated the docetaxel effects, an anticancer used to treat breast cancer, non-small cell lung cancer, hormone-refractory prostate cancer, gastric adenocarcinoma, and squamous cell carcinoma. (96) demonstrated that Mlt enhanced docetaxel's effect in suppressing the growth of breast cancer in an allograft tumor.

The non-specific cell lysis effect of melittin constitutes a considerable challenge to benefit from its anti-cancer properties. To reduce cell lysis and maintain the Mlt apoptotic effects, Holle and coworkers used an MMP2-cleavable Melittin/avidin conjugate specifically targeting tumor ovarian cancer cells SKOV3 and prostate cancer cells DU145 in vitro and in vivo. This conjugation resulted in rendering the peptide inactive until cleaved at the MMP2 site: Hance, microvascular endothelial cells, and proliferation tumor cell suppression (111).

Recently we have recourse to nanoparticle-based drug delivery systems, which show many advantages in cancer treatment, such as good pharmacokinetics, precise targeting of tumor cells, reduction of side effects, and drug resistance (275). Binieka and team proved the effectiveness of carbon nanoparticles as noncarriers, especially UDD (nanodiamond), GN (pristine graphene), and nGO (nanographene oxide). These nanocarriers significantly resulted in glioma grade IV U87 cell death than Mlt itself (276). Likewise, Daniluk and collaborators

demonstrated that the Mlt complex with nGO has a stronger toxic effect on breast cancer cells than Mlt alone. Besides, UDD can protect cells against the lytic effects of Mlt (84).

As we mentioned above, the early stages of melanoma, stage 0, stage 1, and stage 3 could be treated by surgery as benign stages and with medical devices such as transdermal patches to deliver medication through the skin into the bloodstream. This strategy will constitute our subsequent investigation after developing an appropriate formulation and experimentation on animal models.

Table 16. One-way analysis of variances (ANOVA) of outputs of HBV chemical characterization and biological activities results (n = 5).

Variables	Factor-Regions	Mean	SD	F(2,12)	p
In vitro cytotoxic activity of the HBV samples (GI_{50} in µg/mL)					
HepG2	NE	3.87	1.12	3.609	0.059
	C	3.56	0.88		
	S	7.36	4.05		
NCI-H460	NE	6.93	2.37	2.960	0.090
	C	7.15	1.74		
	S	10.96	4.16		
HeLa	NE	3.67	0.99	3.792	0.053
	C	4.29	0.91		
	S	6.15	2.17		
MCF-7	NE	4.03	0.81	5.252	0.023*
	C	5.73	1.12		
	S	8.11	3.16		
MM127	NE	3.44	0.79	5.126	0.025*
	C	4.45	0.66		
	S	5.89	1.83		
PLP2	NE	13.85	8.64	0.982	0.40
	C	13.79	1.58		
	S	19.95	10.68		
Anti-inflammatory activity (EC_{50} in µg/mL)					
RAW264.7	NE	4.98	0.72	6.673	0.01*
	C	6.83	0.92		
	S	10.74	4.24		
Chemical characterization of HBV samples by LC-ESI/MS^n (µg/mL)					
Apamin	NE	2.05	0.14	1.137	0.35
	C	1.99	0.09		
	S	1.80	0.43		
PLA2	NE	7.66	1.13	4.025	0.046*
	C	6.17	1.54		
	S	5.14	1.50		
Melittin	NE	71.71	5.06	0.561	0.585
	C	71.06	3.01		
	S	75.66	11.45		
Metal Determination in HBV Samples					
Ca (mg/g)	NE	1.90	0.83	8.42	0.00*
	C	2.96	0.97		
	S	1.01	0.22		
Zn (mg/g)	NE	1.14	0.15	3.63	0.05*
	C	1.27	0.20		
	S	1.01	0.06		
K (mg/g)	NE	2.87	0.51	6.93	0.01*
	C	2.38	0.67		
	S	1.69	0.16		
Na (mg/g)	NE	1,34	0.22	6.52	0.01*
	C	1.43	0.22		
	S	0.82	0.05		
Mg (mg/g)	NE	0.43	0.11	4.82	0,02*
	C	0.43	0.12		
	S	0.27	0.02		
Pb (ppm)	NE	7.19	1.82	5,64	0.01*
	C	4.27	0.69		
	S	4,59	1.73		

Table 17. Pearson's correlation between the main component HBV (Melittin, PLA2, and apamin) and cytotoxicity and anti-inflammatory activities GI50/EC50 (µg/mL)

	HepG2	NCI-H460	HeLa	MCF-7	MM127	PLP2	RAW264.7
Apamin	-0.497	-0.490	-0.409	-0.430	-0.483	-0.053	-0.560*
PLA2	-0.412	-0.443	-0.391	-0.555*	-0.590*	-0.382	-0.652**
Melittin	-0.311	-0.302	-0.284	-0.214	-0.205	0.154	-0.161

*, significant correlation at the (0.05) level; **, significant correlation at (0.01 level)

Chapter V: Conclusion and Future Perspectives.

This work presented for the first time the chemical and bioactive characterization of *Apis mellifera intermissa* venom. Its main chemical contents, including melittin, PLA2, and apamin, were similar to previous studies on other species. However, we noted moderate distinctions between regions. Overall, *Apis mellifera intermissa* venom showed cytotoxic and anti-inflammatory potential on all studied cell lines. Samples from Northeastern Morocco displayed the best anti-inflammatory and anticancer activities. Excellent antiproliferative properties have been shown essentially on MM127 and MCF-7. The highest percentage of apamin and PLA2 and a moderate melittin content might explain these bioactive features. Some conditions can be pollution sources, affecting the raw material quality, including mine closeness, pesticide use, and collection processes not respecting the good apicultural practices (GAP) of HBV collection. Melittin, PLA2, and apamin, as the main HBV components, can be used as key elements in the standardization and authentication of this product. NIR and AAS can be highly recommended as two reliable techniques for limit tests and authentication as pharmaceutical raw material. According to the findings, *Apis mellifera intermissa* venom from Northeastern Morocco can be considered raw material for our next project strategy. Even though this product showed no hepatotoxicity, it is a risk to claim that this data is sufficient to confirm that this can have a dynamic effect on breast cancer or melanoma. For this purpose, we intend to expand the toxicology studies in vitro and in vivo on various cells and organs, mainly those of the central nervous system, gastrointestinal tract, cardiovascular system, and kidney function, in addition to conducting preclinical studies on animal models. Pharmacotechnique studies will also be performed to establish the most adaptable formulation for honey bee venom pharmaceutical use.

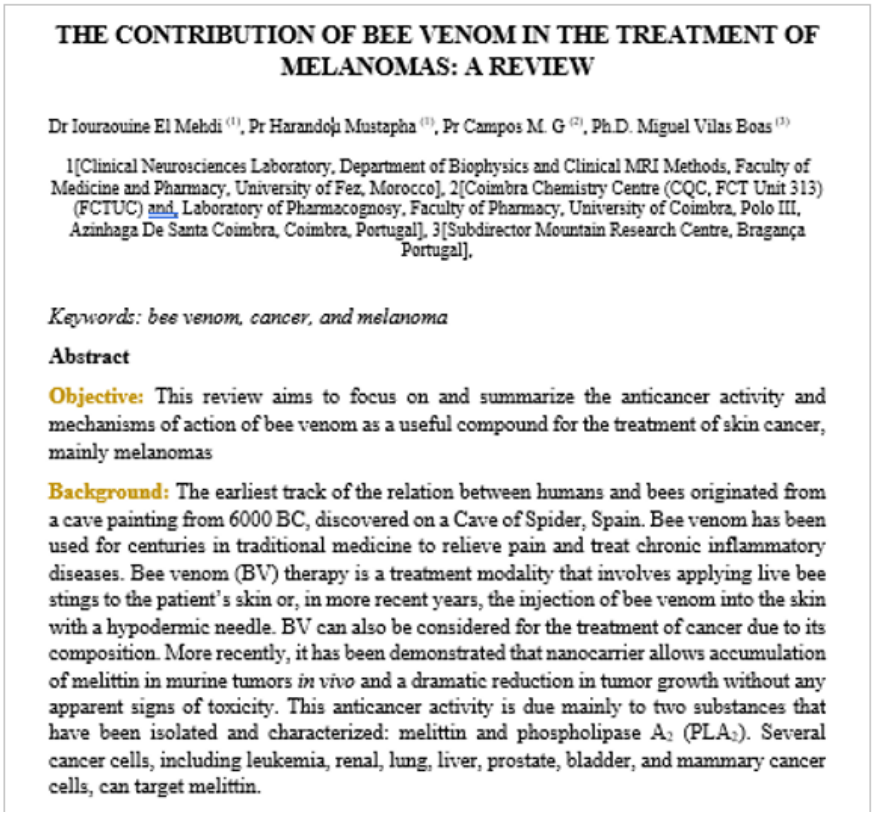
11 Scientific participation, publications, and communications :

Annexes:

Annex 1: The 2nd Congress of the International Federation of Apitherapy. Kaunas-Lithuania/held on September 23rd-26th, 2016.

"The contribution of Bee Venom in the Melanoma Treatment: A review."

Oral communication.



THE CONTRIBUTION OF BEE VENOM IN THE TREATMENT OF MELANOMAS: A REVIEW

Dr Iouraouine El Mehdi [1], Pr Harandou Mustapha [1], Pr Campos M. G [2], Ph.D. Miguel Vilas Boas [3]

1[Clinical Neurosciences Laboratory, Department of Biophysics and Clinical MRI Methods, Faculty of Medicine and Pharmacy, University of Fez, Morocco], 2[Coimbra Chemistry Centre (CQC, FCT Unit 313) (FCTUC) and, Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Coimbra, Polo III, Azinhaga De Santa Coimbra, Coimbra, Portugal], 3[Subdirector Mountain Research Centre, Bragança Portugal],

Keywords: bee venom, cancer, and melanoma

Abstract

Objective: This review aims to focus on and summarize the anticancer activity and mechanisms of action of bee venom as a useful compound for the treatment of skin cancer, mainly melanomas

Background: The earliest track of the relation between humans and bees originated from a cave painting from 6000 BC, discovered on a Cave of Spider, Spain. Bee venom has been used for centuries in traditional medicine to relieve pain and treat chronic inflammatory diseases. Bee venom (BV) therapy is a treatment modality that involves applying live bee stings to the patient's skin or, in more recent years, the injection of bee venom into the skin with a hypodermic needle. BV can also be considered for the treatment of cancer due to its composition. More recently, it has been demonstrated that nanocarrier allows accumulation of melittin in murine tumors *in vivo* and a dramatic reduction in tumor growth without any apparent signs of toxicity. This anticancer activity is due mainly to two substances that have been isolated and characterized: melittin and phospholipase A_2 (PLA_2). Several cancer cells, including leukemia, renal, lung, liver, prostate, bladder, and mammary cancer cells, can target melittin.

Annex 2: The 45th Apimondia International Apicultural Congress. Istanbul-Turkey/held on September 29th-October 4th, 2017.

"NO Bees; No Life" Chapter book, Bee product in the pharmacy.

Book signing ceremony.

Annex 3 : V Congresso Ibérico de Apicultura.

1 a 3 Fevereiro 2018 Coimbra – Portugal.

"A Double-faced Bee Venom Collector: A New Contribution to Improve Apitoxin Collection."

Poster.

El Mehdi, Iouraouine, et al. "A doubled-faced bee venom collector: a new contribution to improve bee venom collection." V Congresso Ibérico de Apicultura. 2018.

P. 2.07 A DOUBLE-FACED BEE VENOM COLLECTOR: A NEW CONTRIBUTION TO IMPROVE APITOXIN COLLECTION

Iouraouine El Mehdi[1]*, Harandou Mustapha[2], Miguel Vilas-Boas[3], Maria Campos[4]

[1]Faculty of Medicine and Pharmacy of Fez Morocco
[2]Clinical Neurosciences Laboratory, Faculty of Medicine and Pharmacy of Fez Morocco
[3]Mountain Research Centre, Polytechnic Institute of Bragança
[4]Faculty of Pharmacy of University of Coimbra

* iouraouine@gmail.com

Annex 4: The 5th International Symposium on Bee Products and International Honey Commission Meeting.
Malta/held on May 7th-10th, 2019.
"A report of Metal Determination in Morocco Honey Bee (*Apis mellifera intermissa*) Venom by Atomic Absorption Spectrometry."
Oral communication.

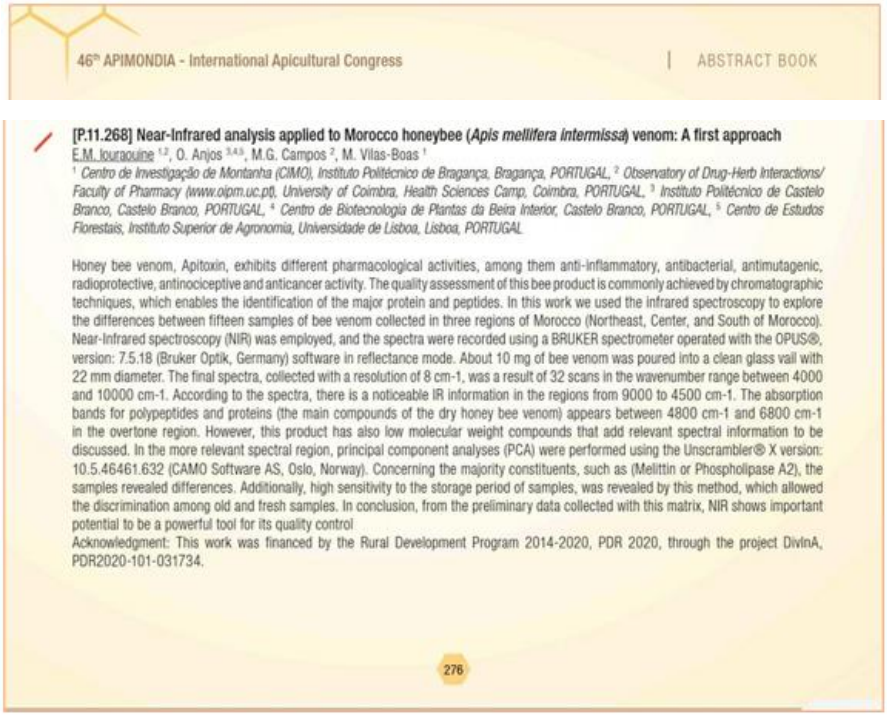
A Report of Metals Determination in Moroccan Honey Bee (*Apis mellifera intermissa*) Venom by Atomic Absorption Spectrometry

Iouraouine El Mehdi[1], Falcão Soraia I[2], Campos Maria Graça[3], Cabral David[2], Vilas-Boas Miguel[2]

1. Faculty of Medicine and Pharmacy of the University of Fez Morocco. 2. Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Portugal. 3. Faculdade de Farmácia da Universidade de Coimbra, Pólo das Ciências da Saúde , Portugal.

Honey bee venom show interesting pharmacological properties. Thence, it is used as raw material in pharmaceutical product manufacturing under several names. Thereby, bee venom or apitoxin, as a biological product, must be submitted to the applicable pharmaceutical regulation. Assessing metal amounts, and particularly the most toxic, is a crucially important issue. Honey bees can be contaminated within foraging, water drinking, polluting industry or even fertilizers used in agriculture. Besides, the collecting process can also be a source of contamination. This work reports the results

Annex 5: The 46th Apimondia-International Apicultural Congress. Montreal/ held on September 8th-12th 2019. Québec-Canada.
"Near-Infrared Analysis Applied to Morocco Honey Bee (*Apis mellifera intermissa*) Venom: A First Approach."
Poster.



46th APIMONDIA - International Apicultural Congress | ABSTRACT BOOK

[P.11.268] Near-Infrared analysis applied to Morocco honeybee (*Apis mellifera intermissa*) venom: A first approach

E.M. Iouraouine [1,2], O. Anjos [3,4,5], M.G. Campos [2], M. Vilas-Boas [1]

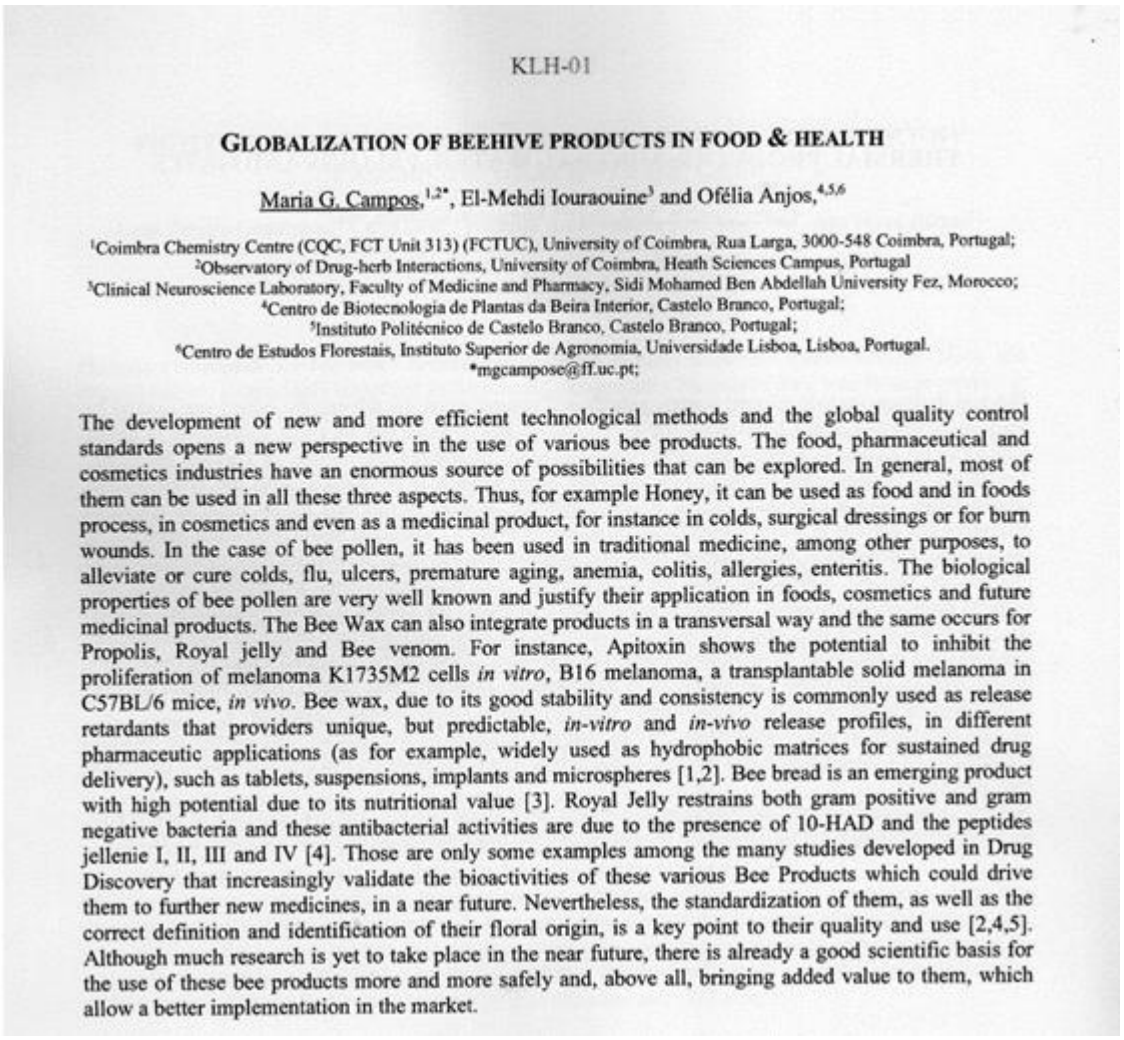
[1] *Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, PORTUGAL,* [2] *Observatory of Drug-Herb Interactions/ Faculty of Pharmacy (www.oipm.uc.pt), University of Coimbra, Health Sciences Camp, Coimbra, PORTUGAL,* [3] *Instituto Politécnico de Castelo Branco, Castelo Branco, PORTUGAL,* [4] *Centro de Biotecnologia de Plantas da Beira Interior, Castelo Branco, PORTUGAL,* [5] *Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, PORTUGAL*

Honey bee venom, Apitoxin, exhibits different pharmacological activities, among them anti-inflammatory, antibacterial, antimutagenic, radioprotective, antinociceptive and anticancer activity. The quality assessment of this bee product is commonly achieved by chromatographic techniques, which enables the identification of the major protein and peptides. In this work we used the infrared spectroscopy to explore the differences between fifteen samples of bee venom collected in three regions of Morocco (Northeast, Center, and South of Morocco). Near-Infrared spectroscopy (NIR) was employed, and the spectra were recorded using a BRUKER spectrometer operated with the OPUS®, version: 7.5.18 (Bruker Optik, Germany) software in reflectance mode. About 10 mg of bee venom was poured into a clean glass vail with 22 mm diameter. The final spectra, collected with a resolution of 8 cm-1, was a result of 32 scans in the wavenumber range between 4000 and 10000 cm-1. According to the spectra, there is a noticeable IR information in the regions from 9000 to 4500 cm-1. The absorption bands for polypeptides and proteins (the main compounds of the dry honey bee venom) appears between 4800 cm-1 and 6800 cm-1 in the overtone region. However, this product has also low molecular weight compounds that add relevant spectral information to be discussed. In the more relevant spectral region, principal component analyses (PCA) were performed using the Unscrambler® X version: 10.5.46461.632 (CAMO Software AS, Oslo, Norway). Concerning the majority constituents, such as (Melittin or Phospholipase A2), the samples revealed differences. Additionally, high sensitivity to the storage period of samples, was revealed by this method, which allowed the discrimination among old and fresh samples. In conclusion, from the preliminary data collected with this matrix, NIR shows important potential to be a powerful tool for its quality control

Acknowledgment: This work was financed by the Rural Development Program 2014-2020, PDR 2020, through the project DivInA, PDR2020-101-031734.

276

Annex 6: The 1st International Online Conference on Natural Products Application: Health, Cosmetic, and Food. Braganca/Portugal. Held on February 4th-5th 2021. "Globalization of Beehive Products in Food and Health" Oral communication.



KLH-01

GLOBALIZATION OF BEEHIVE PRODUCTS IN FOOD & HEALTH

Maria G. Campos,[1,2*], El-Mehdi Iouraouine[3] and Ofélia Anjos,[4,5,6]

[1]Coimbra Chemistry Centre (CQC, FCT Unit 313) (FCTUC), University of Coimbra, Rua Larga, 3000-548 Coimbra, Portugal;
[2]Observatory of Drug-herb Interactions, University of Coimbra, Heath Sciences Campus, Portugal
[3]Clinical Neuroscience Laboratory, Faculty of Medicine and Pharmacy, Sidi Mohamed Ben Abdellah University Fez, Morocco;
[4]Centro de Biotecnologia de Plantas da Beira Interior, Castelo Branco, Portugal;
[5]Instituto Politécnico de Castelo Branco, Castelo Branco, Portugal;
[6]Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade Lisboa, Lisboa, Portugal.
*mgcampose@ff.uc.pt;

The development of new and more efficient technological methods and the global quality control standards opens a new perspective in the use of various bee products. The food, pharmaceutical and cosmetics industries have an enormous source of possibilities that can be explored. In general, most of them can be used in all these three aspects. Thus, for example Honey, it can be used as food and in foods process, in cosmetics and even as a medicinal product, for instance in colds, surgical dressings or for burn wounds. In the case of bee pollen, it has been used in traditional medicine, among other purposes, to alleviate or cure colds, flu, ulcers, premature aging, anemia, colitis, allergies, enteritis. The biological properties of bee pollen are very well known and justify their application in foods, cosmetics and future medicinal products. The Bee Wax can also integrate products in a transversal way and the same occurs for Propolis, Royal jelly and Bee venom. For instance, Apitoxin shows the potential to inhibit the proliferation of melanoma K1735M2 cells *in vitro*, B16 melanoma, a transplantable solid melanoma in C57BL/6 mice, *in vivo*. Bee wax, due to its good stability and consistency is commonly used as release retardants that providers unique, but predictable, *in-vitro* and *in-vivo* release profiles, in different pharmaceutic applications (as for example, widely used as hydrophobic matrices for sustained drug delivery), such as tablets, suspensions, implants and microspheres [1,2]. Bee bread is an emerging product with high potential due to its nutritional value [3]. Royal Jelly restrains both gram positive and gram negative bacteria and these antibacterial activities are due to the presence of 10-HAD and the peptides jellenie I, II, III and IV [4]. Those are only some examples among the many studies developed in Drug Discovery that increasingly validate the bioactivities of these various Bee Products which could drive them to further new medicines, in a near future. Nevertheless, the standardization of them, as well as the correct definition and identification of their floral origin, is a key point to their quality and use [2,4,5]. Although much research is yet to take place in the near future, there is already a good scientific basis for the use of these bee products more and more safely and, above all, bringing added value to them, which allow a better implementation in the market.

References
[1] Silva, D. N. A.; João J. S. S.; Campos M. G. (2014). In Traditional & Folk Herbal Medicine: Recent Researches. Chap. 18, 2014. 541-562. Editor V.K. Gupta, Daya Publishing House. ISBN-10: 8170358744, ISBN-13: 978-8170358749.
[2] Campos MG, Louraouli EM, Anjos O, Bee products in pharmacy. In No Bees no Live. Editors, Peter Kozmus, Boštjan Noč, Karolina Vrtačnik, Beebooks, d.o.o., Žirovnica, Slovenia. 2017. 152-161.
[3] Mărgăoan R., Strant M., Varadi A., Topal E., Yücel B., Cornea-Cipcigan M, Campos M.G. and Vodnar D.C. Antioxidants 8, 568; (2019) 1-40.
[4] Saboor Ahmad, Campos MG , Filippo Fratini, Solomon Zewdu, Altaye, Jianke Li . Int. J. Mol. Sci. 21, 2020, 382-408.
[5] Sajadimajd S, Bahramsoltani R, Iranpanah A, Kumar Patra J, Das G, Gouda S, Rahimi R, Rezaeiamiri E, Cao H, Giampieri F, Battino M, Tundis R, Campos MG, Farzaei MH, Xiao J, Pharmacological Research.151, 104584. (2020). 1-14.

Acknowledgments
M.G. Campos thanks to the financing program UIDP/QUI/00313/2020 and O. Anjos to (UIDB/00239/2020

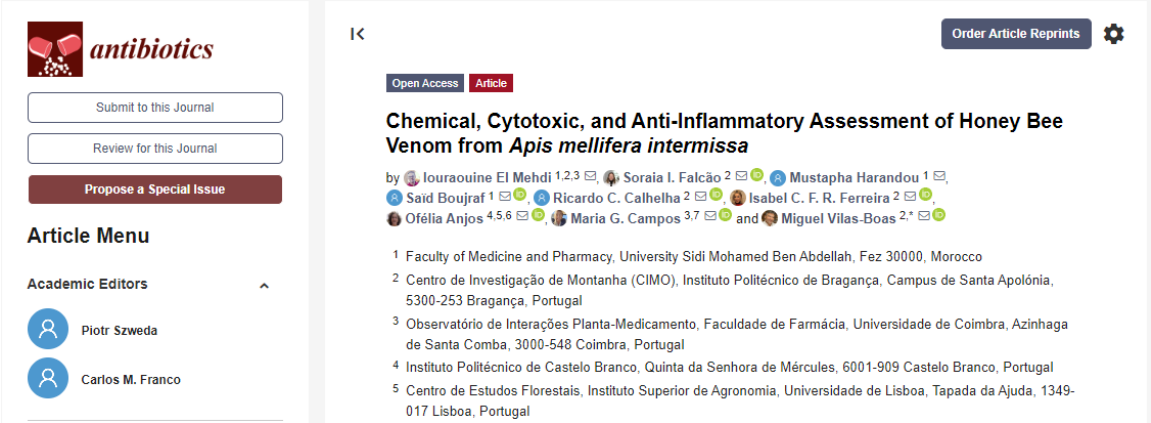
Formations:

Formation1: "1º Seminário de cromatografia: Cromatografia líquida e detectores.", que decorreu no dia 6 de fevereiro de 2019, na Escola Superior Agrária, do Instituto Politécnico de Bragança.

Formation 2: Scientific Writing Workshop-General Principals for Obtaining a Scientific Publication. Porto/Portugal June 6th, 2019.

Paper Publications :

Paper 1: El Mehdi, Iouraouine, et al. "Chemical, cytotoxic, and anti-inflammatory assessment of honey bee venom from Apis mellifera intermissa." Antibiotics 10.12 (2021): 1514.



Paper 2: El Mehdi, Iouraouine, et al. "Analytical methods for honeybee venom characterization." Journal of Advanced Pharmaceutical Technology & Research 13.3 (2022): 154.

Paper 3: Duarte, Diana, et al. "Honeybee venom synergistically enhances the cytotoxic effect of CNS drugs in HT-29 colon and MCF-7 breast cancer cell lines." Pharmaceutics 14.3 (2022): 511.

pharmaceutics

Submit to this Journal

Review for this Journal

Propose a Special Issue

Article Menu

Academic Editors

Jung Min Shin

Eun Sook Lee

Order Article Reprints

Open Access Article

Honeybee Venom Synergistically Enhances the Cytotoxic Effect of CNS Drugs in HT-29 Colon and MCF-7 Breast Cancer Cell Lines

by Diana Duarte [1,2], Soraia I. Falcão [3], Iouraouine El Mehdi [3], Miguel Vilas-Boas [3] and Nuno Vale [1,4,*]

[1] OncoPharma Research Group, Center for Health Technology and Services Research (CINTESIS), Rua Doutor Plácido da Costa, 4200-450 Porto, Portugal

[2] Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

[4] Department of Community Medicine, Health Information and Decision (MEDCIDS), Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal

* Author to whom correspondence should be addressed.

12 Bibliography :

1. Gupta RK, Johan WR, Gupta A, Aspects T. Beekeeping for Poverty Alleviation and Livelihood Security. Springer. Vol. 1. 2014. 670 p.
2. Ilyasov RA, Lee M lyeol, Takahashi J ichi, Kwon HW, Nikolenko AG. A revision of subspecies structure of western honey bee Apis mellifera. Saudi J Biol Sci [Internet]. 2020;27(12):3615–21. Available from: https://doi.org/10.1016/j.sjbs.2020.08.001
3. Cornuet J, Daoudi A, Mohssine EH, Cornuet J, Daoudi A, Mohssine EH, et al. Biometrical Study of Honeybee Populations from Morocco. Apidologie. 1988;19(4):355–66.
4. Ruttner F. Biogeography and Taxonomy of Honeybees. Springer-V. 1988. 284 p.
5. Surendra NS, Jayaram GN, Reddy MRS, Ravikumar H. Comparative morphometric studies of the sting apparatus of the worker bees of four different Apis species (Apis dorsata, Apis mellifera, Apis cerana and Apis florea). J Apic Res. 2013;52(2):74–80.
6. Carreck NL, Andree M, Brent CS, Cox-Foster D, Dade HA, Ellis JD, et al. Standard methods for Apis mellifera anatomy and dissection. J Apic Res. 2013;52(4):0–40.
7. 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.
8. Baracchi D, Francese S, Turillazzi S. Beyond the antipredatory defense: Honey bee venom function as a component of social immunity. Toxicon. 2011 Nov 1;58(6–7):550–7.
9. Yoon S-Y, Kwon Y-B, Kim H-W, Roh D-H, Seo H-S, Han H-J, et al. Bee venom injection produces a peripheral anti-inflammatory effect by activation of a nitric oxide-dependent spinocoeruleus pathway. Vol. 430, Neuroscience Letters. 2008.
10. Raghuraman H, Chattopadhyay A. Melittin: A membrane-active peptide with diverse functions. Biosci Rep. 2007;27(4–5):189–223.
11. Chen J, Guan SM, Sun W, Fu H. Melittin, the Major Pain-Producing Substance of Bee Venom. Neurosci Bull. 2016;
12. Alvarez-Suarez JM. Bee products - chemical and biological properties. Bee Products

- Chemical and Biological Properties. 2017. 1–306 p.

13. Abd El-Wahed AA, Khalifa SAM, 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.
14. Senji Laxme RR, Suranse V, Sunagar K. Arthropod venoms: Biochemistry, ecology, and evolution. Toxicon. 2019;158(November 2018):84–103.
15. Kocyigit A, Metin E, Kaleli S. Anti-inflammatory and antioxidative properties of honey bee venom on Freund s Complete Adjuvant-induced arthritis model in rats. Toxicon. 2019;161(October 2018):4–11.
16. Yang W, Hu F, Xu X. Bee venom and SARS-CoV-2. Toxicon. 2020;181(April):69–70.
17. El-Hanoun A, El-komy A, El-sabrout K, Abdella M. Effect of bee venom on reproductive performance and immune response of male rabbits. Physiol Behav. 2020;223(May):112987.
18. Gajski G, Garaj-Vrhovac V. Melittin: A lytic peptide with anticancer properties. Environ Toxicol Pharmacol. 2013 Sep 1;36(2):697–705.
19. Purification O, Derived M. One-Step Purification of Melittin Derived from Apis mellifera Bee Venom. 2017;27:84–91.
20. Matysiak 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(2016):69–76.
21. Domerego PR. La thérapie au venin d'abeille. France; 2015.
22. Dempsey CE. The actions of melittin on membranes. 1990;1031:143–61.
23. Popplewell JF, Swann MJ, Freeman NJ, Mcdonnell C, Ford RC. Quantifying the effects of melittin on liposomes. 2007;1768:13–20.
24. Naito A, Kawamura I, Javkhlantugs N. Recent Solid-State NMR Studies of Membrane-Bound Peptides and Proteins. 1st ed. Annual Reports on NMR Spectroscopy. Elsevier Ltd.; 2015. 333–411 p.
25. Terwilligert TC, Eisenbergg D. The Structure of Melittin. 1982;257(11):6016–22.
26. Zhou J, Zhao J, Zhang S, Shen J, Qi Y, Xue X, et al. Quantification of melittin and

apamin in bee venom lyophilized powder from Apis mellifera by liquid chromatography-diode array detector-tandem mass spectrometry. Anal Biochem. 2010;404(2):171–8.

27. Er C. Structure and Membrane Interactions of Antimicrobial Peptides as Viewed by Solid-State NMR Spectroscopy. Annu Reports NMR Spectrosc. 2008;63(07):1–21.
28. Eun J, Kumar V, Lee E, Oh M, June J. Melittin e A bee venom component e Enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion. J Pharmacol Sci. 2019;140(1):26–32.
29. Postma TL. Neurotoxic Animal Poisons and Venoms. First Edit. Clinical Neurotoxicology. Elsevier; 2009. 463–489 p.
30. Okumura M, Shimamoto S, Hidaka Y. A chemical method for investigating disulfide-coupled peptide and protein folding. FEBS J. 2012;279(13):2283–95.
31. Palma MS. Hymenoptera Insect Peptides. Second Edi. Handbook of Biologically Active Peptides. Elsevier Inc.; 2013. 416–422 p.
32. Bystrov VF, Okhanov V V., Miroshnikov AI, Ovchinnikov YA. Solution spatial structure of apamin as derived from NMR study. FEBS Lett. 1980;119(1):113–7.
33. Demonchauxl, P; Ganellin, CR; Dunn, PM; Haylett, DG; Jenkinson D. Search for the Pharmacophore of the K+ Channel Blocker, Apamin. Eur J Med Chem. 1991;(26):915–20.
34. Lee YM, Cho SN, Son E, Song CH, Kim DS. Apamin from bee venom suppresses inflammation in a murine model of gouty arthritis. J Ethnopharmacol. 2020;257(April):112860.
35. Bodnaryk RP. Structure And Function Of Insect Peptides. Annu Rev Entomol. 1978;13(1):69–132.
36. Dotimas EM, Hamid KR, Hider RC, Ragnarsson U. Isolation and structure analysis of bee venom mast cell degranulating peptide. Biochim Biophys Acta (BBA)/Protein Struct Mol. 1987;911(3):285–93.
37. Banks BEC, 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(2):350–4.

38. Matysiak J, Schmelzer CEH, Neubert RHH, Kokot ZJ. Characterization of honeybee venom by MALDI-TOF and nanoESI-QqTOF mass spectrometry. J Pharm Biomed Anal. 2011;54(2):273–8.

39. El-seedi HR, El-wahed AAA, Sheikh BY, Shaden A. Bee Venom Composition ; Unravelling The Biodiversity And The Potential Biological Activities.

40. dos Santos-Pinto JRA, Perez-Riverol A, Lasa AM, Palma MS. Diversity of peptidic and proteinaceous toxins from social Hymenoptera venoms. Toxicon. 2018 Jun 15;148:172–96.

41. Lee KS, Kim BY, Yoon HJ, Choi YS, Jin BR. Secapin, a bee venom peptide, exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities. Dev Comp Immunol. 2016;63(2016):27–35.

42. Mourelle D, Brigatte P, Bringanti LDB, 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.

43. Chen J, Lariviere WR. The nociceptive and anti-nociceptive effects of bee venom injection and therapy : A double-edged sword. Prog Neurobiol [Internet]. 2010;92:151–83. Available from: www.elsevier.com/locate/pneurobio%0AThe

44. Koburova SS and K. Adolapin-A Newly Isolated Analgesic and Antiinflammatory Polypeptide from Bee Venom. Toxicon. 1982;20(1):317–21.

45. Jun Chena, b and WRL. The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword Jun. Prog Neurobiol. 2010;92(2):151–83.

46. Xu X, Nelson JW. Solution Structure of Tertiapin Determined Using Nuclear Magnetic Resonance and Distance Geometry. ProteinsStructure, Funct Genet. 1993;137:124–37.

47. Jin W, Lu Z. Synthesis of a stable form of tertiapin: A high-affinity inhibitor for inward-rectifier K+ channels. Biochemistry. 1999;38(43):14286–93.

48. Doupnik CA. Venom-derived peptides inhibiting Kir channels: Past, present, and

future. Neuropharmacology. 2017;127(2017):161–72.

49. Ramu Y, Xu Y, Lu Z. Engineered specific and high-affinity inhibitor for a subtype of inward-rectifier K+ channels. Proc Natl Acad Sci U S A. 2008;105(31):10774–8.

50. Miroshnikov AI, Boĭkov VA, Snezhkova LG, Severin SE, Shvets VI. Interaction of tertiapin, a neurotoxin from bee venom, with calmodulin. Vol. 9, Bioorganicheskaia khimiia. 1983. p. 26–32.

51. Peck ML, O'Connor R. Procamine and Other Basic Peptides in the Venom of the Honeybee (Apis mellifera). J Agric Food Chem. 1974;22(1):51–3.

52. Banks BEC, Shipolini RA. Chemistry and pharmacology of honey-bee venom. Venoms of the Hymenoptera: Biochemical, pharmacological, and behavioral aspects. ACADEMIC PRESS INC. (LONDON) LTD; 1986. 329–416 p.

53. Lowy PH, Sarmiento L, Mitchell HK. Polypeptides minimine and melittin from bee venom: Effects on Drosophila. Arch Biochem Biophys. 1971;145(1):338–43.

54. Shipolini R a, Callewaert GL, Cottrell RC, Vernon C a. The amino-acid sequence and carbohydrate content of phospholipase A2 from bee venom. Eur J Biochem. 1974;48(2):465–76.

55. Lai CC, Her GR. Analysis of N-glycosylation of phospholipase A2 from the venom of individual bees by microbore high-performance liquid chromatography-electrospray mass spectrometry using an ion trap mass spectrometer. J Chromatogr B. 2002;766:243–50.

56. Kuchler K, Gmachl M, Sippl MJ, Kreil G. Analysis of the cDNA for phospholipase A2 from honeybee venom glands. Eur J Biochem. 1989;184:249–54.

57. Hossen MS, Shapla UM, Gan SH, Khalil MI. Impact of bee venom enzymes on diseases and immune responses. Molecules. 2017;22(1).

58. Lee G, Bae H. Bee venom phospholipase A2: Yesterday's enemy becomes today's friend. Toxins (Basel). 2016;8(2).

59. Chris Hopley, Tony Bristow, Anneke Lubben, Alec Simpson, Elaine Bull, Katerina Klagkou, Julie Herniman JL. Towards a universal product ion mass spectral library - reproducibility of product ion spectra across eleven different mass spectrometers.

Rapid Commun Mass Spectrom. 2008;22(12):1779–86.

60. Li L, Sun C, Freeby S, Yee D, Kieffer-Jaquinod S, Guerrier L, et al. Protein Sample Treatment with Peptide Ligand Library: Coverage and Consistency. J Proteomics Bioinform. 2009;02(12):485–94.

61. Six DA, Dennis EA. The expanding superfamily of phospholipase A2 enzymes: Classification and characterization. Biochim Biophys Acta - Mol Cell Biol Lipids. 2000;1488(1–2):1–19.

62. Annand RR, Kontoyianni M, Penzotti JE, Dudler T, Lybrand TP, Gelb MH. Active Site of Bee Venom Phospholipase A 2 : The Role of Histidine-34 ,. 1996;4591–601.

63. Burke JE, Dennis EA. Phospholipase A 2 structure/function, mechanism, and signaling. J Lipid Res. 2009;50(Supplement):S237–42.

64. Owen MD. Relationship Between Age and Hyaluronidase Activity in the Venons of Queen and Worker Honey Bees (Apis Mellifera L .). 1979;17:94–8.

65. 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(8):3569–73.

66. Bala E, Hazarika R, Singh P, Yasir M, Shrivastava R. A biological overview of Hyaluronidase: A venom enzyme and its inhibition with plants materials. Mater Today Proc [Internet]. 2018;5(2):6406–12. Available from: https://doi.org/10.1016/j.matpr.2017.12.252

67. Padavattan S. Crystal structure determination of hyaluronidase, a major bee venom allergen, in complex with an IgG Fab fragment and purification and biophysical characterization of bovine testes hyaluronidase. University of Basel; 2006.

68. Marković-Housley Z, Miglierini G, Soldatova L, Rizkallah PJ, Müller U, Schirmer T. Crystal structure of hyaluronidase, a major allergen of bee venom. Structure. 2000;8(10):1025–35.

69. Li J, Wang Z, Tan K, Qu Y, Nieh JC. Effects of natural and synthetic alarm pheromone and individual pheromone components on foraging behavior of the giant Asian honey bee, Apis dorsata. J Exp Biol. 2014;217(19):3512–8.

70. Nouvian M, Reinhard J, Giurfa M. The defensive response of the honeybee Apis

mellifera. J Exp Biol. 2016;219(22):3505–17.

71. Hider RC. Honeybee venom. Bee World. 1989;70(2):53–4.

72. Boch. R, Shearer. D. A SBC. Identification of Iso-Amyl Acetat as an Active Component in the Sting Pheromone of the Honey Bee. Nature. 1962;196:1179–81.

73. 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(1):163–75.

74. Kokot ZJ, Matysiak J. Inductively coupled plasma mass spectrometry determination of metals in honeybee venom. J Pharm Biomed Anal. 2008;48(3):955–9.

75. Pont E, Christiane D. Controle des Impuretes dans les substances pour Usage Pharmaceutique selon la Pharmacopee Europeenne: Evolution gdes Connaissance et des Methodes Analytiques de Controle. 2011.

76. Pharmacopée Européenne. Guide technique pour L ' ÉLABORATION DES MONOGRAPHIES. 2015.

77. Heinen TE, Gorini da Veiga AB. Arthropod venoms and cancer. Toxicon. 2011;57(4):497–511.

78. Moon DO, Park SY, Heo MS, Kim KC, Park C, Ko WS, et al. Key regulators in bee venom-induced apoptosis are Bcl-2 and caspase-3 in human leukemic U937 cells through downregulation of ERK and Akt. Int Immunopharmacol. 2006;6(12):1796–807.

79. Son DJ, Lee JW, Lee YH, Song HS, Lee CK, Hong JT. Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. Pharmacol Ther. 2007;115(2):246–70.

80. Moga MA, Dimienescu OG, Arvătescu CA, Ifteni P, Pleş L. Anticancer activity of toxins from bee and snake venom-an overview on ovarian cancer. Molecules. 2018;23(3):1–21.

81. Upadhyay RK. Use of Animal Venom Peptides / Toxins in Cancer Therapeutics. Curr Trends Biomed Eng Biosci. 2018;16(4).

82. Wehbe R, Frangieh J, Rima M, Obeid D El, Obeid E. Bee Venom : Overview of Main

Compounds and Bioactivities for Therapeutic Interests. Molecules. 2019;24(24):1–13.

83. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar 4;144(5):646–74.
84. Daniluk K, Kutwin M, Grodzik M, Wierzbicki M, Strojny B, Szczepaniak J, et al. Use of Selected Carbon Nanoparticles as Melittin Carriers for MCF-7 and MDA-MB-231 Human Breast Cancer Cells. Materials (Basel). 2020;13(90):1–20.
85. Welsh JE. Animal Models for Studying Prevention and Treatment of Breast Cancer. Animal Models for the Study of Human Disease. Elsevier; 2013. 997–1018 p.
86. Comşa Ş, Cîmpean AM, Raica M. The story of MCF-7 breast cancer cell line: 40 Years of experience in research. Anticancer Res. 2015;35(6):3147–54.
87. Sharkawi FZ El, Saleh SS, Sayed AFM El. Potential Anti-Cancer Activity of Snake Venom, Bee Venom and Their Components in Liver and Breast Carcinoma. Int J Pharm Sci Res. 2015;6(8):0–6.
88. Khamis AAA, Ali EMM, El-Moneim MAA, Abd-Alhaseeb MM, El-Magd MA, Salim EI. Hesperidin, piperine, and bee venom synergistically potentiate the anticancer effect of tamoxifen against breast cancer cells. Biomed Pharmacother. 2018 Sep 1;105(2018):1335–43.
89. Aka JA, Lin S. Comparison of Functional Proteomic Analyses of Human Breast Cancer Cell Lines T47D and MCF7. PLoS One. 2012;7(2):1–9.
90. Jeong Y, Choi Y, Shin J, Cho H, Kang J, Park K, et al. Melittin suppresses EGF-induced cell motility and invasion by inhibiting the PI3K / Akt / mTOR signaling pathway in breast cancer cells. FOOD Chem Toxicol. 2014;68:218–25.
91. Jung Hyun Park, Yun-Jeong Jeong, Kwan-Kyu Park, Hyun-Ji Cho, Il-Kyung Chung, Kwan-Sik Min, Mihyun Kim, Kwang-Gill Lee, Joo-Hong Yeo, Kwang-Kyun Park Y-CC. Melittin Suppresses PMA-Induced Tumor Cell Invasion by Inhibiting NF- κ B and AP-1-Dependent MMP-9 Expression. Mol Cells. 2010;29:209–15.
92. M. Golubovskaya V. Focal Adhesion Kinase as a Cancer Therapy Target. Anticancer Agents Med Chem. 2011;10(10):735–41.

93. Rigiracciolo DC, Santolla MF, Lappano R, Vivacqua A, Cirillo F, Galli GR, et al. Focal adhesion kinase (FAK) activation by estrogens involves GPER in triple-negative breast cancer cells. J Exp Clin Cancer Res. 2019;38(1):1–16.
94. Jung GB, Huh J-E, Lee H-J, Kim D, Lee G-J, Park H-K, et al. Anti-cancer effect of bee venom on human MDA-MB-231 breast cancer cells using Raman spectroscopy. Biomed Opt Express. 2018;9(11):5703.
95. Loibl S, Gianni L. HER2-positive breast cancer. Lancet. 2017;389(10087):2415–29.
96. Duffy C, Sorolla A, Wang E, Golden E, Woodward E, Davern K, et al. Honeybee venom and melittin suppress growth factor receptor activation in HER2-enriched and triple-negative breast cancer. npj Precis Oncol. 2020;4(1).
97. Kong GM, Tao WH, Diao YL, Fang PH, Wang JJ, Bo P, et al. Melittin induces human gastric cancer cell apoptosis via activation of the mitochondrial pathway. World J Gastroenterol. 2016;22(11):3186–95.
98. El-Bassiony MN, Mahfouz HM, Hussein AS, El-Hamamy MM, Abdel Daim MM, Bufo SA. Effect of honey bee venom on cancer in rats model. J Entomol. 2016;13(3):72–83.
99. Badria F, Fathy H, Fatehe A, Elimam D, Ghazy M. Evaluate the cytotoxic activity of honey, propolis, and bee venom from different localities in Egypt against liver, breast, and colorectal cancer. J Apitherapy. 2017;2(1):1.
100. Ceremuga M, Stela M, Janik E, Gorniak L, Synowiec E, Sliwinski T, et al. Melittin—a natural peptide from bee venom which induces apoptosis in human leukemia cells. Biomolecules. 2020;10(2):1–14.
101. Sobolewski C, Cerella C, Dicato M, Ghibelli L, Diederich M. The Role of Cyclooxygenase-2 in Cell Proliferation and Cell Death in Human Malignancies. Int J cell biology. 2010;2010.
102. Moon DO, Park SY, Choi YH, Kim ND, Lee C, Kim GY. Melittin induces Bcl-2 and caspase-3-dependent apoptosis through downregulation of Akt phosphorylation in human leukemic U937 cells. Toxicon. 2008;51(1):112–20.

103. Li J, Yuan J. Caspases in apoptosis and beyond. Oncogene. 2008;27(2008):6194–206.

104. Chen S, Li X, Qin J, Chen Y, Liu L, Zhang D, et al. Honeybee venom possesses anticancer and antiviral effects by differential inhibition of HPV E6 and E7 expression in cervical cancer. Int J Clin Exp Med. 2015;8(7):10548–57.

105. Ip SW, Wei HC, Lin JP, Kuo HM, Liu KC, Hsu SC, et al. Bee venom induced cell cycle arrest and apoptosis in human cervical epidermoid carcinoma Ca Ski cells. Anticancer Res. 2008;28(2 A):833–42.

106. Oršolić N. Potentiation of Bleomycin Lethality in HeLa and V79 Cells by Bee Venom. 2009;(20):317–26.

107. Zarrinnahad H, Mahmoodzadeh A, Hamidi MP, Mahdavi M, Moradi A, Bagheri KP, et al. Apoptotic Effect of Melittin Purified from Iranian Honey Bee Venom on Human Cervical Cancer HeLa Cell Line. Int J Pept Res Ther [Internet]. 2017;24(4):1–9. Available from: http://dx.doi.org/10.1007/s10989-017-9641-1

108. Alizadehnohi M, Nabiuni M, Nazari Z, Safaeinejad Z, Irian S. The synergistic cytotoxic effect of cisplatin and honey bee venom on human ovarian cancer cell line A2780cp. J Venom Res [Internet]. 2012;3:22–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23301148%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3522379

109. Amini E, Baharara J, Nikdel N, Abdollahi FS. Cytotoxic and Pro-Apoptotic Effects of Honey Bee Venom and Chrysin on Human Ovarian Cancer Cells. Asia Pacific Journal Med Toxicol. 2015;4(2):68–73.

110. Alonezi S, Tusiimire J, Wallace J, Dufton MJ, Parkinson JA, Young LC, et al. Metabolomic profiling of the synergistic effects of melittin in combination with cisplatin on ovarian cancer cells. Metabolites. 2017;7(2).

111. Holle L, Song W, Holle E, Wei Y, Wagner T, Yu X. A matrix metalloproteinase 2 cleavable melittin/avidin conjugate specifically targets tumor cells in vitro and in vivo. Int J Oncol. 2003;22(1):93–8.

112. Jo M, Park MH, Kollipara PS, An BJ, Song HS, Han SB, et al. Anti-cancer effect of bee

venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. Toxicol Appl Pharmacol. 2012;258(1):72–81.

113. TM Xu, MH Cui MS-CJLD. Inhibitory effect of melittin on ovarian cancer. Chinese J Lab Diagnosis. 2007;8(013).
114. Su M, Chang W, Zhang K, Cui M, Wu S, Xu T. Expression and purification of recombinant ATF-mellitin a new type fusion protein targeting ovarian cancer cells, in P. pastoris. Oncol Rep. 2016;35(2):1179–85.
115. Chung Won Lee, Sung Jong Lee, Eun Kyung Park, Yong Seok Lee, Joo Hee Yoon, Soo Young Hur MJS. Antitumor activity of melittin in ovarian cancer cell lines. J Korean Soc Obstet Gynecol. 2012;367–367.
116. Guven KN, Sevim H, Keskin N. Purified melittin from honeybee Apis mellifera venom's effects on U118 glioblastoma cell line. J Biotechnol. 2018 Aug 30;280:S72.
117. Ip SW, Chu YL, Yu CS, Chen PY, Ho HC, Yang JS, et al. Bee venom induces apoptosis through intracellular Ca 2+-modulated intrinsic death pathway in human bladder cancer cells. Int J Urol. 2012;19(1):61–70.
118. Park MH, Choi MS, Kwak DH, Oh KW, Yoon DY, Han SB, et al. Anti-Cancer Effect of Bee Venom in Prostate Cancer Cells Through Activation of Caspase Pathway Via Inactivation of NF-κB. Prostate. 2011;71(8):801–12.
119. Hye JP, Seong HL, Dong JS, Ki WO, Ki HK, Ho SS, et al. Antiarthritic effect of bee venom: Inhibition of inflammation mediator generation by suppression of NF-κB through interaction with the p50 subunit. Arthritis Rheum. 2004;50(11):3504–15.
120. Ji H, Ju D, Woo C, Suk M, Soo U, Sueb H, et al. Melittin inhibits inflammatory target gene expression and mediator generation via interaction with IkB kinase. Biochem Pharmacol [Internet]. 2007;73(2):237–47. Available from: http://dx.doi.org/10.1016/j.bcp.2006.09.023
121. Moon D-O, Park S-Y, Lee K-J, Heo M-S, Kim K-C, Kim M-O, et al. Bee venom and melittin reduce proinflammatory mediators in lipopolysaccharide-stimulated BV2 microglia. Int Immunopharmacol. 2007;7(8):1092–101.

122. Jang H-S, Kim SK, Han J-B, Ahn H-J, Bae H, Min B-I. Effects of bee venom on the pro-inflammatory responses in RAW264.7 macrophage cell line. Vol. 99, Journal of Ethnopharmacology. 2005.

123. Lee JD, Kim SY, Kim TW, Lee SH, Yang HI, Lee DI, et al. Anti-inflammatory effect of bee venom on type II collagen-induced arthritis. Am J Chin Med. 2004;32(3):361–7.

124. Kim WH, An HJ, Kim JY, Gwon MG, Gu H, Jeon M, et al. Anti-inflammatory effect of melittin on porphyromonas gingivalis LPS-stimulated human keratinocytes. Molecules. 2018;23(2).

125. Cordeiro I, Saad F, Morato F, Luiza M, Beatriz S, Mello V De. Anti-inflammatory effect of bee venom on antigen-induced arthritis in rabbits : Influence of endogenous glucocorticoids. J Ethnopharmacol. 2010;130(1):175–8.

126. Snyder LM, Fortier NL, Trainor J, Jacobs J, Leb L, Lubin B, et al. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. J Clin Invest. 1985;76(5):1971–7.

127. Rekka E, Kourounakis L, Kourounakis P. Antioxidant activity of and interleukin production affected by honey bee venom. Arzneimittel-Forschung/Drug Res. 1990;40(8):912–3.

128. Han SM, Lee KG, Yeo JH, Oh BY, Kim BS, Lee W, et al. Effects of honeybee venom supplementation in drinking water on growth performance of broiler chickens. Poult Sci. 2010;89(11):2396–400.

129. Sobral F, Sampaio A, Falcão S, Queiroz MJRP, 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.

130. Gu H, An H, Kim J, Kim W, Gwon M, Kim H, et al. Bee venom attenuates Porphyromonas gingivalis and RANKL-induced bone resorption with osteoclastogenic differentiation. Food Chem Toxicol. 2019;129(April):344–53.

131. Zolfagharian H, Mohajeri M, Babaie M. Bee venom (Apis mellifera) an effective potential alternative to gentamicin for specific bacteria strains-Bee venom an

effective potential for bacteria. J Pharmacopuncture. 2016;19(3):225–30.

132. Sarhan M, El-Bitar AMH, Hotta H. Potent virucidal activity of honeybee “Apis mellifera” venom against Hepatitis C Virus. Toxicon. 2020;188(September):55–64.

133. Dantas CG, Nunes TLGM, Nunes TLGM, da Paixão AO, Reis FP, de L. Júnior W, et al. Pharmacological evaluation of bee venom and melittin. Brazilian J Pharmacogn. 2014;24(1):67–72.

134. Yamasaki SC, Mendes MT, Alponti RF, Silveira PF. Efficacy of parenteral administration of bee venom in experimental arthritis in the rat: A comparison with methotrexate. Toxicon [Internet]. 2015;98:75–88. Available from: http://dx.doi.org/10.1016/j.toxicon.2015.02.016

135. Awad K, Abushouk AI, AbdelKarim AH, Mohammed M, Negida A, Shalash AS. Bee venom for the treatment of Parkinson’s disease: How far is it possible? Biomed Pharmacother. 2017 Jul 1;91(2017):295–302.

136. Alvarez-Fischer D, Noelker C, Vulinović F, Grünewald A, Chevarin C, Klein C, et al. Bee Venom and Its Component Apamin as Neuroprotective Agents in a Parkinson Disease Mouse Model. PLoS One. 2013;8(4):2–9.

137. Salthun-Lassalle B, Hirsch EC, Wolfart J, Ruberg M, Michel PP. Rescue of mesencephalic dopaminergic neurons in culture by low-level stimulation of voltage-gated sodium channels. J Neurosci. 2004;24(26):5922–30.

138. Toulorge D, Guerreiro S, Hild A, Maskos U, Hirsch EC, Michel PP. Neuroprotection of midbrain dopamine neurons by nicotine is gated by cytoplasmic Ca^{2+}. FASEB J. 2011;25(8):2563–73.

139. Wolfart J, Neuhoff H, Franz O, Roeper J. Differential expression of the small-conductance, calcium-activated potassium channel SK3 is critical for pacemaker control in dopaminergic midbrain neurons. J Neurosci. 2001;21(10):3443–56.

140. Deignan J, Luján R, Bond C, Riegel A, Watanabe M, Williams JT, et al. SK2 and SK3 expression differentially affect firing frequency and precision in dopamine neurons. Neuroscience. 2012;217:67–76.

141. Bond CT, Herson PS, Strassmaier T, Hammond R, Stackman R, Maylie J, et al. Small

conductance Ca2+-activated K+ channel knock-out mice reveal the identity of calcium-dependent afterhyperpolarization currents. J Neurosci. 2004;24(23):5301–6.

142. Mohamed WA, Abd-elhakim YM, Ismail SAA. Involvement of the anti-inflammatory, anti-apoptotic, and anti-secretory activity of bee venom in its therapeutic effects on acetylsalicylic acid-induced gastric ulceration in rats. Toxicology. 2019;419(February):11–23.
143. Hozzein WN, Badr G, Badr BM, Allam A, Al A, Al-wadaan MA, et al. Bee venom improves diabetic wound healing by protecting functional macrophages from apoptosis and enhancing Nrf2, Ang-1, and Tie-2 signaling. Mol Immunol [Internet]. 2018;103(April):322–35. Available from: https://doi.org/10.1016/j.molimm.2018.10.016
144. Badr G, Hozzein WN, Badr BM, Al Ghamdi A, Saad Eldien HM, Garraud O. Bee Venom Accelerates Wound Healing in Diabetic Mice by Suppressing Activating Transcription Factor-3 (ATF-3) and Inducible Nitric Oxide Synthase (iNOS)-Mediated Oxidative Stress and Recruiting Bone Marrow-Derived Endothelial Progenitor Cells. J Cell Physiol. 2016;231(10):2159–71.
145. Son DJ, Ha SJ, Song HS, Lim Y, Yun YP, Lee JW, et al. Melittin inhibits vascular smooth muscle cell proliferation through induction of apoptosis via suppression of nuclear factor-κB and Akt activation and enhancement of apoptotic protein expression. J Pharmacol Exp Ther. 2006;317(2):627–34.
146. Holmes D. The Cancer That Rises With The Sun. Nature. 2014;515(2014):2011–2.
147. Herlyn M. Human melanoma: Development and progression. Cancer Metastasis Rev. 1990;9(2):101–12.
148. Bethesda M. Melanoma Treatment for Health Professional Version [Internet]. National Cancer Institute. 2021. p. 1–78. Available from: https://www.cancer.gov/types/skin/hp/melanoma-treatment-pdq

149. Medical TACS. What Is Melanoma Skin Cancer ? [Internet]. American Cancer Society. 2019. p. 1–14. Available from: Written by%0AThe American Cancer Society mwww.cancer.org/cancer
150. Gershenwald JE, Scolyer RA, Hess KR, Faries MB, Kirkwood JM, Mcarthur GA. Melanoma Staging: Evidence-Based Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual. CA CancerJ Clin. 2018;67(6):472–92.
151. Garg M, Couturier D, Nsengimana J, Fonseca NA, Wongchenko M, Yan Y, et al. Tumor gene expression signature in primary melanoma predicts long-term outcomes. Nat Commun. 2021;12(2021):1–14.
152. WILD BWS and CP. World Cancer Report 2014. 2014.
153. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020 : GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. Cacancer J. 2021;0(0):1–41.
154. Society AC. Cancer Facts & Figures 2021. 2021.
155. Lakraichi MA, Cles M, Superieur PDE. Le profil épidémiologique du mélanome cutané dans la région de Marrakech. 2007.
156. Bouskoul M, Amal S. Le profil épidémiologique du mélanome cutané dans la région d ' Agadir. 2009.
157. Tarwate M, Benchikhi H, Adarmouch L, Benider A, Amine M, Zamiati S, et al. Case series. Pan Afr Med J. 2014;18(44):2–6.
158. Pelosi JB. Understanding Melanoma A guide for people with cancer, their families, and friends. 2021. 58 p.
159. MedlinePlus. Melanoma [Internet]. 2021. p. 1–6. Available from: https://medlineplus.gov/melanoma
160. Dermatology AA of. MELANOMA : SIGNS AND SYMPTOMS [Internet]. AAD. 2021. p. 1–10. Available from: https://www.aad.org/public/diseases/skin-cancer/types/common/melanoma/symptoms
161. Abbasi NR, Shaw HM, Rigel DS, Friedman RJ, McCarthy WH, Osman I, et al. Early Diagnosis of Cutaneous Melanoma. Jama. 2004;292(22):2771.

162. Corona R, Mele A, Amini M, De Rosa G, Coppola G, Piccardi P, et al. Interobserver variability on the histopathologic diagnosis of cutaneous melanoma and other pigmented skin lesions. J Clin Oncol. 1996;14(4):1218–23.
163. Farmer ER, Gonin R, Hanna MP. Discordance in the histopathologic diagnosis of melanoma and melanocytic nevi between expert pathologists. Hum Pathol. 1996;27(6):528–31.
164. Button-Sloan A, Hardy M, Henderson M, Gyorki DE, Fishburn P, Soyer HP, et al. Diagnosis and management of cutaneous melanoma. Aust J Gen Pract. 2020;49(11):733–9.
165. Ng JC, Swain S, Dowling JP, Wolfe R, Simpson P, Kelly JW. The impact of partial biopsy on histopathologic diagnosis of cutaneous melanoma: Experience of an Australian tertiary referral service. Arch Dermatol. 2010;146(3):234–9.
166. Fink C, Haenssle HA. Non-invasive tools for the diagnosis of cutaneous melanoma. Ski Res Technol. 2017;23(3):261–71.
167. Allan C. Halpern, Ashfaq A. Marghoob OR. Melanoma Treatment [Internet]. Skin Cancer Foundation. 2021. p. 1–7. Available from: https://www.skincancer.org/skin-cancer-information/melanoma/
168. Institut NC. Melanoma Treatment (PDQ®)–Patient Version [Internet]. National Cancer Institute. 2020. p. 1–27. Available from: ttps://www.cancer.gov/types/skin
169. Cancer.Net. Melanoma : Types of Treatment The cancer care team [Internet]. ASCO. 2020. p. 1–14. Available from: https://www.cancer.net/cancer-types/melanoma
170. Gerstt JE, Salomon Y. Inhibition by Melittin and Fluphenazine of Melanotropin Receptor Function and Adenylate Cyclase in M2R Melanoma Cell Membranes. Endocrinology. 1987;12(5):1766–72.
171. Liu X, Chen D, Xie L, Zhang R. Effect of honey bee venom on the proliferation of K1735M2 mouse melanoma cells in-vitro and growth of murine B16 melanomas in-vivo. J Pharm Pharmacol. 2002;54:1083–9.
172. Tu W-C, Wu C-C, Hsieh H-L, Chen C-Y, Hsu S-L. Honeybee venom induces calcium-

dependent but caspase-independent apoptotic cell death in human melanoma A2058 cells. Toxicon. 2008;52(2):318–29.

173. Pacáková V, Štulík K, Thi Hau P, Jelínek I, Vinš I, Sýkora D. Comparison of high-performance liquid chromatography and capillary electrophoresis for the determination of some bee venom components. J Chromatogr A. 1995;700(1–2):187–93.
174. Banks BEC, Dempsey CE, Pearce FL, Vernon CA, Wholley TE. New methods of isolating bee venom peptides. Anal Biochem. 1981 Sep 1;116(1):48–52.
175. Rybak-Chmielewska H, Szczęsna T. HPLC study of the chemical composition of honeybee (Apis mellifera L.) venom. J Apic Sci. 2004;48(2):103–9.
176. Gauldie J, Hanson JM, Rumjanek FD, Shipolini RA, Vernon CA. The Peptide Components of Bee Venom. Eur J Biochem. 1976;61(2):369–76.
177. Marz R, Mollay C, Kreil G, Zelger J. Queen bee venom contains much less phospholipase than worker bee venom. Insect Biochem. 1981;11(6):685–90.
178. 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(7):813–20.
179. 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(1):59–65.
180. Baudou FG, Fusco L, Giorgi E, Diaz E, Municoy S, Desimone MF, et al. Physicochemical and biological characterization of nano venoms, a new tool formed by silica nanoparticles and Crotalus durissus terrificus venom. Colloids Surfaces B Biointerfaces. 2020;193(January):111128.
181. Altmann F, Kubelka V, Staudacher E, Uhl K, März L. Characterization of the isoforms of phospholipase A2 from honeybee venom. Insect Biochem. 1991;21(5):467–72.
182. LAWRENCE SC-Ib and A. High Resolution of Honey Bee (Apis Mellifera) Venom Peptides by Propionic Acid/Urea Polyacrylamide Gel Electrophoresis After Ethanol Precipitation. Toxicon. 1989;27(7):781–7.
183. Szókán GY, Horváth J, Almás M, Saftics GY, Palócz A. Liquid Chromatographic

Analysis and Separation of Polypeptide Components from Honey Bee Venoms. J Liq Chromatogr. 1994;17(16):3333–49.

184. Ameratunga RV, Hawkins R, Prestidge R, Marbrook J. A high-efficiency method for purification and assay of bee venom phospholipase A2. Pathology. 1995;27(2):157–60.

185. Mahmoodzadeh A, Zarrinnahad H, Bagheri KP, Moradia A, Shahbazzadeh D. First report on the isolation of melittin from Iranian honey bee venom and evaluation of its toxicity on gastric cancer AGS cells. J Chinese Med Assoc. 2015;78(10):574–83.

186. Nowar EE. Venom Glands Parameters, Venom Production and Composition of Honeybee Apis mellifera L . Affected by Substitute Feeding. 2016;596–603.

187. Dong J, Ying B, Huang S, Ma S. High-performance liquid chromatography combined with intrinsic fluorescence detection to analyze melittin in individual honeybee (Apis mellifera) venom sac. J Chromatogr B. 2015;1002:139–43.

188. Francese S, Lambardi D, Mastrobuoni G. Detection of Honeybee Venom in Envenomed Tissues by Direct MALDI MSI. Am Soc Mass Spectrosc. 2009;20:112–23.

189. Jinhui. Zhou, Jing. Zhao, Suxia. Zhang, Jianzhong. Shen, Yitao. Qi, Xiaofeng. Xue, Yi. Li, Liming. Wu, Jinzhen. Zhang, Fang. Chen LC. Quantification of melittin and apamin in bee venom lyophilized powder from Apis mellifera by liquid chromatography – diode array detector – tandem mass spectrometry. Anal Biochem. 2010;404(2010):171–8.

190. Kokot ZJ, Matysiak J, Urbaniak B, Dereziński P. New CZE-DAD method for honeybee venom analysis and standardization of the product. Anal Bioanal Chem. 2011;399(7):2487–94.

191. Ionete RE, Dinca OR, Tamaian R. Exploring Apis Mellifera Venom Compounds Using Highly Efficient Methods. Prog Cryog Isot Sep. 2013;16(2):89–100.

192. Matysiak J, Hajduk J, Pietrzak Ł, Schmelzer CEH, Kokot ZJ. Shotgun proteome analysis of honeybee venom using targeted enrichment strategies. Toxicon. 2014;90:255–64.

193. Klupczynska A, Plewa S, Dereziński P, Garrett TJ, Rubio VY, Kokot ZJ, et al.

Identification and quantification of honeybee venom constituents by multiplatform metabolomics. Nat Res. 2020;10(0123456789):1–11.

194. Nassar M. The Potential of Natural Venom of Apis Mellifera for the Control of Grains Weevil Adults (Sitophilus Granarius-Coleopter-. Int J Entomol Res. 2013;01(01).

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

196. Lai C, Her G. Analysis of phospholipase A 2 glycosylation patterns from the venom of individual bees by capillary electrophoresis/electrospray ionization mass spectrometry using an ion trap mass spectrometer. Rapid Commun Mass Spectrom. 2000;14:2012–8.

197. Graaf DC De, Regina M, Braga B, Magalhães RM, Abreu D, Blank S, et al. Standard methods for Apis mellifera venom research. J Apic Res. 2020;0(0):1–31.

198. Lakshmi Priya G, Rameshkumar P, Ponmanickam P. Identification of Volatile and Protein Profiles in the Sting and Mandibular Glands of the Worker Honey Bee (Apis cerana indica). Biochem Physiol. 2013;02(01):1–7.

199. Allen W. Benton RAM and JDS. Venom Collection from Honey Bees. Science (80-) [Internet]. 1963;142(3589):228–30. Available from: http://www.jstor.org/stable/1712211

200. Rybak M, Janina Muszynska, Piotr Skubida JM. A Technology for Bee Venom Collection. 1995;(2):223–31.

201. Michal Rybak PS. Application of Coupled Electrical and Sound Stimulation for Honeybee Venom Collection. J Apic Sci. 2007;51(2):63–6.

202. Nelson DA, O'Connor R. The venom of the honeybee (Apis mellifera): free amino acids and peptides. Can J Biochem. 1968;46(10):1221–6.

203. Shkenderov S. Anaphylactogenic properties of bee venom and its fractions. Toxicon. 1974;12(5):529–34.

204. J. Jentsch HWM. Bee Venom Peptides XVIII. Peptide-M and MCD-Peptide: Isolation

and Characterization. Int J Pept Res. 1977;9:78–9.

205. K.Räder, A.Wildfeuer, F. Wintersberger PB and H-WM. Characterization of bee venom and its main components by high-performance liquid chromatography. J Chromatogr. 1987;408:341–8.

206. 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(7):781–7.

207. Kemeny DM, Dalton N, Lawrence AJ, Pearce FL, Vernon CA. The purification and characterization of hyaluronidase from the venom of the honey bee, Apis mellifera. Eur J Biochem. 1984;139(2):217–23.

208. Pattanaargson S, Roboz J. Determination of hyaluronidase activity in venoms using capillary electrophoresis. Toxicon. 1996;34(10):1107–17.

209. Pacakova V, Stulik K. Validation of a method for determination of phospholipase A2 and melittin in bee venom preparations by capillary electrophoresis. J Aoac Int. 2000;83(3):549–54.

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

211. Tamvakopoulos C. Mass Spectrometry for the Quantification of Bioactive Peptides in Biological Fluids. Mass Spectrom Rev. 2007;26:389–402.

212. Matysiak 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(2016):69–76.

213. Frangieh J, Salma Y, Haddad K, Mattei C, Legros C, Fajloun Z, et al. First characterization of the venom from Apis mellifera syriaca, a honeybee from the Middle East region. Toxins (Basel). 2019;11(4).

214. Francese S, Lambardi D, Mastrobuoni 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(1):112–23.

215. Ferreira Junior RS, Sciani JM, Marques-Porto R, Junior AL, Orsi R de O, Barraviera B,

et al. Africanized honey bee (Apis mellifera) venom profiling: Seasonal variation of melittin and phospholipase A2 levels. Toxicon [Internet]. 2010 Sep 1 [cited 2018 Mar 21];56(3):355–62. Available from: https://www.sciencedirect.com/science/article/pii/S004101011000142X

216. Resende VMF, Vasilj A, Santos KS, Palma MS, Shevchenko A. Proteome and phosphoproteome of Africanized and European honeybee venoms. Proteomics. 2013;13(17):2638–48.
217. Banach P, Suchy W, Dereziński P, Matysiak J, Kokot ZJ, Nowak-Markwitz E. Mass spectrometry as a tool for biomarkers searching in gynecological oncology. Vol. 92, Biomedicine and Pharmacotherapy. 2017.
218. Van Vaerenbergh M, De Smet L, Rafei-Shamsabadi D, Blank S, Spillner E, Ebo DG, et al. IgE recognition of chimeric isoforms of the honeybee (Apis mellifera) venom allergen Api m 10 evaluated by protein array technology. Mol Immunol. 2015;63(2):449–55.
219. Anderson D, Terwilliger TC, Wickner W, Eisenberg D. Melittin forms crystals that are suitable for high-resolution X-ray structural analysis and which reveal a molecular 2-fold axis of symmetry. J Biol Chem. 1980;255(6):2578–82.
220. Terwilliger TC, Weissman L, Eisenberg D. The Structure of Melittin in the Form I Crystals and its Implication for Melittin's Lytic and Surface Activities. Biophys J [Internet]. 1982;37(1):353–61. Available from: http://dx.doi.org/10.1016/S0006-3495(82)84683-3
221. Wilcox W, Eisenberg D. Thermodynamics of melittin tetramerization determined by circular dichroism and implications for protein folding. Protein Sci. 1992;1:641–53.
222. Bello J, Bello HR, Granados E. Conformation and Aggregation of Melittin : Dependence on pH and Concentration +. Biochemistry. 1982;21:461–5.
223. Hristova K, Dempsey CE, White SH. Structure, Location, and Lipid Perturbations of Melittin at the Membrane Interface. Biophys J [Internet]. 2001;80(2):801–11. Available from: http://dx.doi.org/10.1016/S0006-3495(01)76059-6
224. Larry R. Brown, Jürgen Lauter Wein KW. High-Resolution H-NMR Studies of Self-

Aggregation of Melittin in Aqueous Solution. Biochim Biophys Acta -. 1980;622:231–44.

225. Miura Y. NMR studies on the monomer–tetramer transition of melittin in an aqueous solution at high and low temperatures. Eur Biophys Soc Assoc. 2012;41:629–36.
226. Wemmer D, Kallenbach NR, Lett YAF. Structure of Apamin in Solution : A Two-Dimensional Nuclear Magnetic Resonance Study. Biochemistry. 1983;22(8):1901–6.
227. Joseph H. B. Pease DEW. Solution Structure of Apamin Determined by Nuclear Magnetic Resonance and Distance Geometry? 1988;6(1977):8491–8.
228. David L.Scott, Zbyszek Otwinowski, Michael H. Gelb PBS. Crystal Structure of Bee-Venom Phospholipase A2 in a Complex with a Transition-State Analogue. Science (80-). 1990;250:1563–6.
229. Arni R., Ward R. Phospholipase A2—a structural review. Toxicon. 1996;34(8):827–41.
230. Saraswathy N, Ramalingam P, Saraswathy N, Ramalingam P. Protein sequencing techniques. Concepts Tech Genomics Proteomics [Internet]. 2011 Jan 1 [cited 2019 Jan 22];193–201. Available from: https://www.sciencedirect.com/science/article/pii/B9781907568107500146
231. Peiren N, Vanrobaeys F, De Graaf DC, Devreese B, Van Beeumen J, Jacobs FJ. The protein composition of honeybee venom was reconsidered by a proteomic approach. Biochim Biophys Acta - Proteins Proteomics. 2005;1752(1):1–5.
232. Peiren N, de Graaf DC, Vanrobaeys F, Danneels EL, Devreese B, Van Beeumen J, et al. Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage. Toxicon. 2008;52(1):72–83.
233. Kim K Hixson, Daniel Lopez-Ferrer, Errol W Robinson and LP-T. Proteomics. Encycl Spectrosc Spectrom. 2017;766–73.
234. Patil KJ and D. Proteomics. Innovative Approaches in Drug Discovery. Elsevier Inc.; 2017. 273–294 p.
235. Baek JH, Lee SH. Identification and characterization of venom proteins of two

solitary wasps, Eumenes pomiformis and Orancistrocerus drewseni. Toxicon. 2010;56(4):554–62.

236. Van Vaerenbergh M, Debyser G, Devreese B, de Graaf DC. Exploring the hidden honeybee (Apis mellifera) venom proteome by integrating a combinatorial peptide ligand library approach with FTMS. J Proteomics. 2014;99(2014):169–78.
237. Pawlak M, Klupczynska A, Kokot ZJ, Matysiak J. Extending metabolomic studies of Apis mellifera venom: LC-MS-based targeted analysis of organic acids. Toxins (Basel). 2019;12(1):1–13.
238. Blum MS, Fales HM, Tucker KW, Collins AM. Chemistry of the sting apparatus of the worker honeybee. J Apic Res. 1978;17(4):218–21.
239. Weber A, Leopold M, Altmann F. Characteristics of the Asparagine-liked Oligosaccharide from Honeybee Venom Phospholipase A2. Comp Biochem Physiol. 1986;83(2):321–4.
240. Hollander T, Aeed PA, Elhammer P. Characterization of the oligosaccharide structures on bee venom phospholipase A. Carbohydr Res. 1993;247:291–7.
241. Kubelka V, Altma F, Staudacher E, Trelter V, Marz L, Hard K, et al. Primary structures of N-linked carbohydrate chains from honeybee venom phospholipase A2. Eur J Biochem. 1993;213:1193–204.
242. Soldatova, Lyudmila N, Tsai, Chaoming, Dobrovolskaia E, Slater JE. Characterization of the N -glycans of recombinant bee venom hyaluronidase (Api m 2) expressed in insect cells. Allergy Asthma Proc. 2007;28(2):210–5.
243. Nallahali S Surendra, Hemagirigowda Ravikumar MRSR. Evaluation of catecholamines and amino acids from venom reservoir extract of Indian honey bee (Apis) species Evaluation of catecholamines and amino acids from venom reservoir extract of Indian honey bee (Apis) species de Veneno de reserva de especies. J Apic Res. 2014;53(5):514–9.
244. Yang YR, Ren YF, Dong GM, Yang RJ, Liu HX, Du YH, et al. Determination of Methanol in Alcoholic Beverages by Two-dimensional Near-infrared Correlation Spectroscopy Determination of Methanol in Alcoholic Beverages by Two-

Dimensional Near-infrared Correlation Spectroscopy. 2016;2719(May).

245. Alonso-esteban JI, Pinela J, Barros L, Ana Ć, Sokovi M, Calhelha RC, et al. Phenolic composition and antioxidant, antimicrobial, and cytotoxic properties of hop (Humulus lupulus L .) Seeds. 2019;134(April):154–9.

246. Falcão SI, Calhelha RC, Touzani S, Lyoussi B. In Vitro Interactions of Moroccan Propolis Phytochemicals on Human Tumor Cell Lines and Anti-Inflammatory Properties. Biomolecules. 2019;9(315).

247. Abreu RMV, Ferreira ICFR, Calhelha RC, Lima RT, Vasconcelos MH, Adega F, et al. Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-b]pyridine-2- carboxylate derivatives: In vitro evaluation, cell cycle analysis, and QSAR studies. Eur J Med Chem. 2011;46(12):5800–6.

248. Raghuram P, Raju IVS, Sriramulu J. Heavy metals testing in active pharmaceutical ingredients : an alternate approach. Int J Pept Sci. 2010;65(1):15–8.

249. Wollein U, Bauer B, Habernegg R, Schramek N. Potential metal impurities in active pharmaceutical substances and finished medicinal products - A market surveillance study. Eur J Pharm Sci [Internet]. 2015;77:100–5. Available from: http://dx.doi.org/10.1016/j.ejps.2015.05.028

250. Balaram V. Recent advances in the determination of elemental impurities in pharmaceuticals - Status, challenges and moving frontiers. TrAC - Trends Anal Chem [Internet]. 2016;80:83–95. Available from: http://dx.doi.org/10.1016/j.trac.2016.02.001

251. European Medicines Agency. ICH guideline Q3D on elemental impurities. Vol. 44. London, United Kingdom; 2017. p. 1–84.

252. Pasquini C. Near-infrared spectroscopy: A mature analytical technique with new perspectives – A review. Anal Chim Acta [Internet]. 2018;1026:8–36. Available from: https://doi.org/10.1016/j.aca.2018.04.004

253. Anjos O, Santos AJA, Paixão V, Estevinho LM. Physicochemical characterization of Lavandula spp. honey with FT-Raman spectroscopy. Talanta. 2018;178(September

2017):43–8.

254. Barra I, Kharbach M, Qannari EM, Hanafi M, Cherrah Y, Bouklouze A. Predicting cetane number in diesel fuels using FTIR spectroscopy and PLS regression. Vib Spectrosc [Internet]. 2020;111(June):103157. Available from: https://doi.org/10.1016/j.vibspec.2020.103157

255. Jr JMM. Building a Tiered Approach to In Vitro Predictive Toxicity Screening : A Focus on Assays with In Vivo Relevance. Comb Chem High Throughput Screen. 2010;13:188–206.

256. Johansson J, Larsson MH, Hornberg JJ. Predictive in vitro toxicology screening to guide chemical design in drug discovery. Curr Opin Toxicol. 2020;15:99–108.

257. Niles AL, Moravec RA, Riss TL. Update on in vitro cytotoxicity assays for drug development. Drug Discov. 2008;3(6):655–70.

258. Cai L, Qin X, Xu Z, Song Y, Jiang H, Wu Y, et al. Comparison of Cytotoxicity Evaluation of Anticancer Drugs between Real-Time Cell Analysis and CCK - 8 Method. Am Chem Soc. 2019;4:10.1021/acsomega.9b01142 ACS Omega 2019, 4, 12036−.

259. Orellana EA, Kasinski AL. Sulforhodamine B(SRB) Assay in Cell Culture to Investigate Cell Proliferation. Bio Protoc. 2017;6(21).

260. Kuete V, Karaosmanog O, Sivas H. Anticancer Activities of African Medicinal Spices and Vegetables. In: Medicinal Spices and Vegetables from Africa Therapeutic Potential Against Metabolic, Inflammatory, Infectious and Systemic Diseases. 2017. p. 271–97.

261. Hu H, Chen D, Li Y, Zhang X. Effect of polypeptides in bee venom on growth inhibition and apoptosis induction of the human hepatoma cell line SMMC-7721 in-vitro and Balb/c nude mice in-vivo. J Pharm Pharmacol. 2006;58(1):83–9.

262. Huang C, Jin H, Qian Y, Qi S, Luo H, Luo Q. Hybrid Melittin Cytolytic Peptide- Driven Ultrasmall Lipid Nanoparticles Block Melanoma Growth in Vivo. ACSNANO. 2013;

263. Choi KE, Hwang CJ, Gu SM, Park MH, Kim JH, Park JH, et al. Cancer cell growth inhibitory effect of bee venom via increase of death receptor 3 expression and inactivation of NF-kappa B in NSCLC cells. Toxins (Basel). 2014;6(8):2210–28.

264. Jang M, Shin M, Lim S, Han S, Park H, Shin I, et al. Bee Venom Induces Apoptosis and Inhibits Expression of Cyclooxygenase-2 mRNA in Human Lung Cancer Cell Line NCI-H1299. J Pharmacol Sci. 2003;91(2):95–104.

265. Zhang S, Liu Y, Ye Y, Wang X-R, Lin L-T, Xiao L-Y, et al. Bee venom therapy: Potential mechanisms and therapeutic applications. Toxicon. 2018 Jun 15;148:64–73.

266. Yoon S-Y, Kim H-W, Roh D-H, Kwon Y-B, Jeong T-O, Han H-J, et al. The anti-inflammatory effect of peripheral bee venom stimulation is mediated by central muscarinic type 2 receptors and activation of sympathetic preganglionic neurons. Brain Res. 2005;1049(2):210–6.

267. Merly L, Smith SL, Ã LM, Ãã SLS. Murine RAW 264. 7 cell line as an immune target : are we missing something ? Immunopharmacol Immunotoxicol. 2017;39(2):55–8.

268. Cho H-J, Jeong Y-J, Park K-K, Park Y-Y, Chung I-K, Lee K-G, et al. Bee venom suppresses PMA-mediated MMP-9 gene activation via JNK/p38 and NF-κB-dependent mechanisms. J Ethnopharmacol. 2010 Feb 17;127(3):662–8.

269. Lin L, Zhu BP, Cai L. Therapeutic effect of melittin on a rat model of chronic prostatitis induced by Complete Freund's Adjuvant. Biomed Pharmacother. 2017;90(2017):921–7.

270. Gu H, Han SM, Park KK. Therapeutic Effects of Apamin as a Bee Venom Component for Non-Neoplastic Disease. Toxins (Basel). 2020;12(3):1–17.

271. Oršolić N. Bee venom in cancer therapy. Cancer Metastasis Rev. 2012;31(1–2):173–94.

272. Ma R, Mahadevappa R, Kwok HF. Venom-based peptide therapy: insights into anti-cancer mechanism. Oncotarget [Internet]. 2017;8(59):100908–30. Available from: http://www.oncotarget.com/fulltext/21740

273. Abd-Elhakim YM, Khalil SR, Awad A, Al-Ayadhi LY. Combined cytogenotoxic effects of bee venom and bleomycin on rat lymphocytes: An in vitro study. Biomed Res Int. 2014;2014.

274. Sleijfer S. Bleomycin-induced pneumonitis. Chest. 2001;120(2):617–24.

275. Yao Y, Zhou Y, Liu L, Xu Y, Chen Q, Wang Y, et al. Nanoparticle-Based Drug Delivery

in Cancer Therapy and Its Role in Overcoming Drug Resistance. Front Mol Biosci. 2020;7(August):1–14.

276. Binieka P, Bugajska Ż, Daniluk K, Jaworski S. Carbon nanoparticles as transporters of melittin to glioma grade IV U87 cells in vitro model. Anim Sci. 2017;56(1):23–32.