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Honeybees produce important bee products that are beneficial for human and animal health. One of these products is bee venom, whose effectiveness in complementary therapies has been proven by scientific studies. Bee venom is a bee product with many biological properties, produced in the venom sacs of queen and worker bees and used mainly for defense purposes. Bee venom has applications in cosmetics, pharmacology, and Apitherapy. However, it is known that the content and quality of bee venom varies depending on the bee species, collection methods, existing flora, and overall colony status. This study aims to reveal the proteomic and apitherapeutic properties of bee venoms collected from Türkiye, one of the richest countries in the world in terms of endemic plant species and honeybee biodiversity. The analysis of bee venoms obtained from different regions of the country and from different honeybee species and ecotypes shows that all collected venoms have dose-dependent cytotoxic and apoptosis-inducing effects on different cancer cells. According to the analysis results, Kırklareli (*Apis mellifera carnica*), Gökçeada (*A.m. anatoliaca* ecotype) and Efe bee (*A.m. anatoliaca* ecotype) venoms were the most effective on pancreas epithelioid carcinoma (PANC-1); Macahel (*A.m. caucasia*), Farasin (Local ecotype), Kıbrıs Mallıdağ (*A.m. cypria*) venoms were found to be most effective on human prostate adenocarcinoma (PC3) while Adana and Antakya (*A.m. syriaca* local ecotype) venoms appear to be most effective on human cervix adenocarcinoma (HELA) cells. At the same time, the genomic and proteomic sequences of the main protein melittin, which is also an indicator of the apitherapeutic properties of the venom, and the main proteins of the venom were comparatively analyzed in the bee venom and venom gland samples collected within the scope of the study. The analysis results reveal that melittin occurs in varying abundances among species. In conclusion, bee venoms collected from Türkiye, which has an important phytogeographic location, appear to have high and diverse potential for pharmaceutical or apitherapeutic use. This high effect seen in the study results, supported by detailed scientific research, may be predicted as a valuable resource for the world bee venom industry and apitherapy practices.



PROTEOMIC ANALYSIS OF BEE VENOMS FROM DIFFERENT HONEYBEE SPECIES AND THEIR APITHERAPEUTIC EFFECT ON VARIOUS CANCER CELLS

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Introduction

- Bee venom (BV) is a biologically rich natural product that has been used by humans for centuries due to its therapeutic properties. Proteomic analyses have revealed that venoms obtained from different bee species contain numerous peptides, enzymes and biogenic amines, primarily melittin and phospholipase A2.
- Due to these components, bee venom shows promise in various clinical applications through mechanisms such as suppressing cancer cell growth, inducing apoptotic processes, and reducing inflammatory responses.



Aim of the study

- The present study aims to perform a proteomic characterization of bee venoms obtained from different *Apis mellifera* subspecies and to investigate their apitherapeutic potential against various cancer cell lines.
- To determine the biochemical properties of honeybee species and ecotypes naturally found in different geographical regions of Türkiye, which has a rich honeybee and plant biodiversity in terms of venom production.
- At the same time, the genomic and proteomic sequences of the main protein melittin, which is also an indicator of the apitherapeutic properties of the venom and the main proteins of the venom were comparatively analyzed in the bee venom and venom gland samples collected within the scope of the study.
- To provide scientific data on a national basis to increase the bee venom production capacity in Türkiye and to establish national standards and to produce scientific data that will inspire world literature.



Materials and Method



For this purpose, honeybee venom was collected from apiaries located in 51 different locations in Türkiye between May and August, by the same researchers using the same venom collection machine, using electroshock traps on the hive. The samples were then transported to the laboratory under cold chain storage and lyophilized.

Bee race and ecotype determination was performed using geometric morphometry analysis, providing a concrete race/ecotype determination. The protein content (BCA method), cytotoxic potential (MTT method) and apoptotic activity (Flow cytometry method) of bee venom were determined.

Furthermore, the genomic and proteomic sequences of melittin and the main venom proteins were analyzed in bee venom and venom gland samples collected as part of the study.



Bee venom samples were collected using the electro-shock method. Venom glands were collected for genomic analysis from regions with the highest activity and protein content. These regions were areas with pure races or local ecotypes. At the same time, bee samples were collected from all colonies where venom was collected, and the species were identified using geometric morphometry.

Protein content determination: Protein content was determined with bicinchoninic acid (BCA) assay.

Cell culture: The cytotoxic potential of the honey bee venom was demonstrated against human cancerous cells.

In vitro cytotoxicity assay: A modified colorimetric MTT test is used to determine the cytotoxicity of the crude venom.

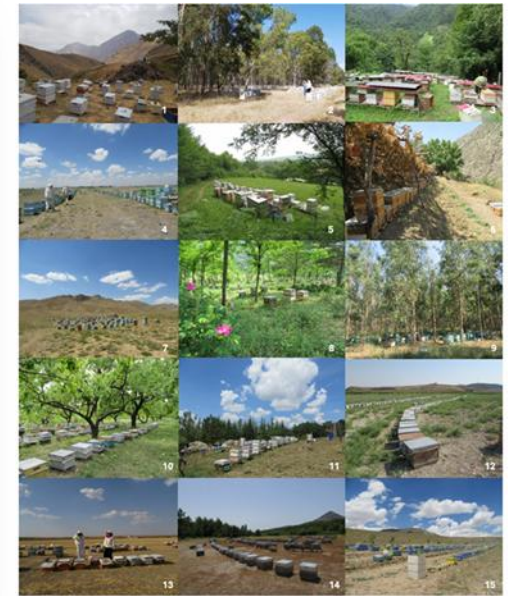
Flow-cytometry analysis for apoptosis: Using flow cytometry methods and the FITC Annexin V Apoptosis Detection Kit I, the presence of apoptotic cells was assessed based on the exposure of phosphatidylserine residues.

SDS - PAGE Analysis: SDS-PAGE was used to control the purity of the complex combination of bee venom proteins by separating them according to their molecular weight.

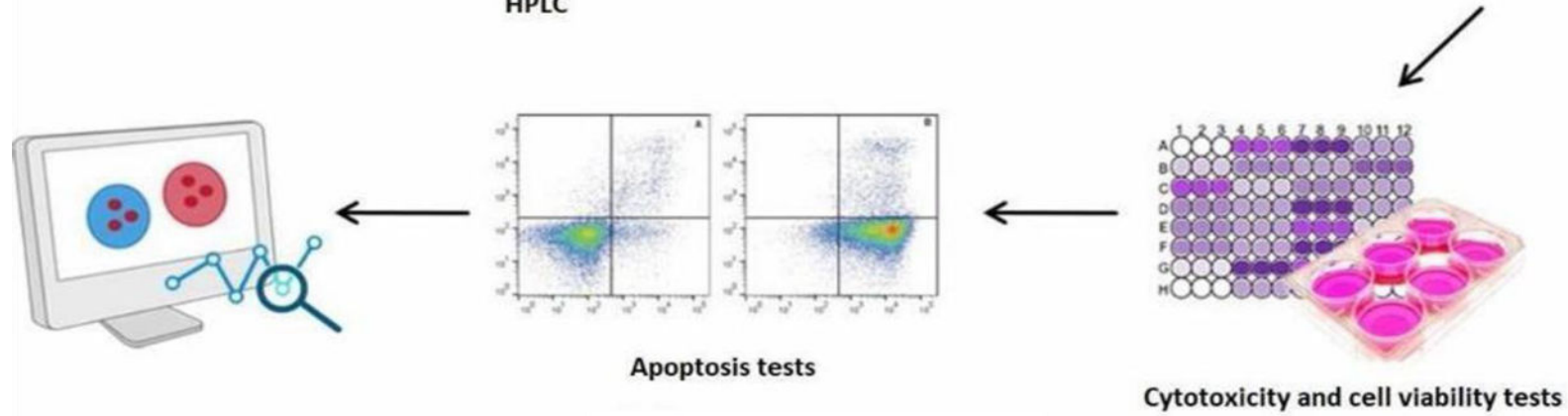
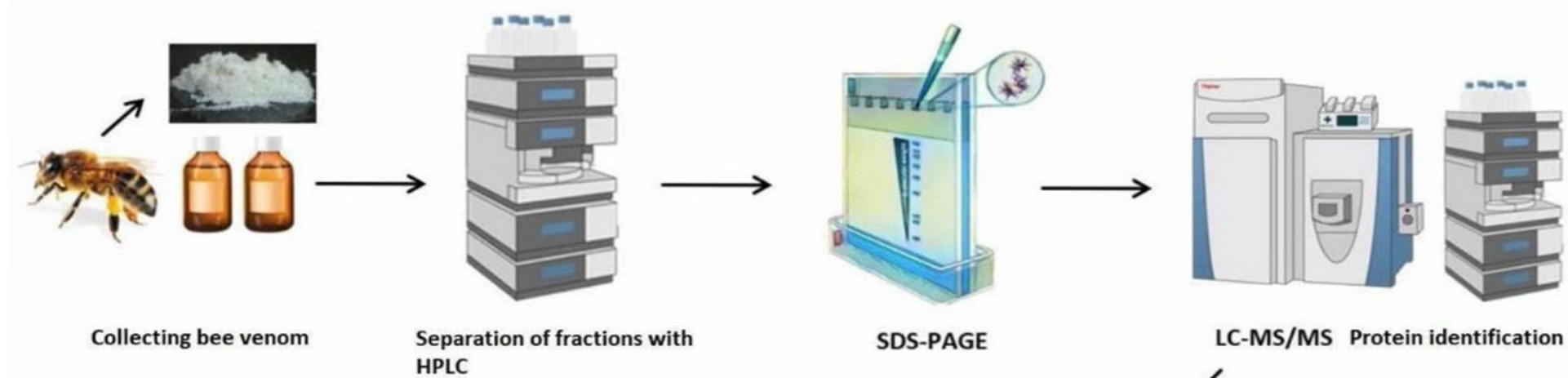
Proteomic analysis: The peptides obtained in the study were separated by liquid chromatography and characterized by LC-MS/MS analysis.

Genomic analysis: Genomic analyses were performed by isolating RNA from venom glands, synthesizing cDNA, and amplifying the melittin CDS from different *Apis mellifera* species and ecotypes. PCR products were verified by Sanger sequencing, and sequence variations were analyzed bioinformatically using Clustal Omega and BLAST.

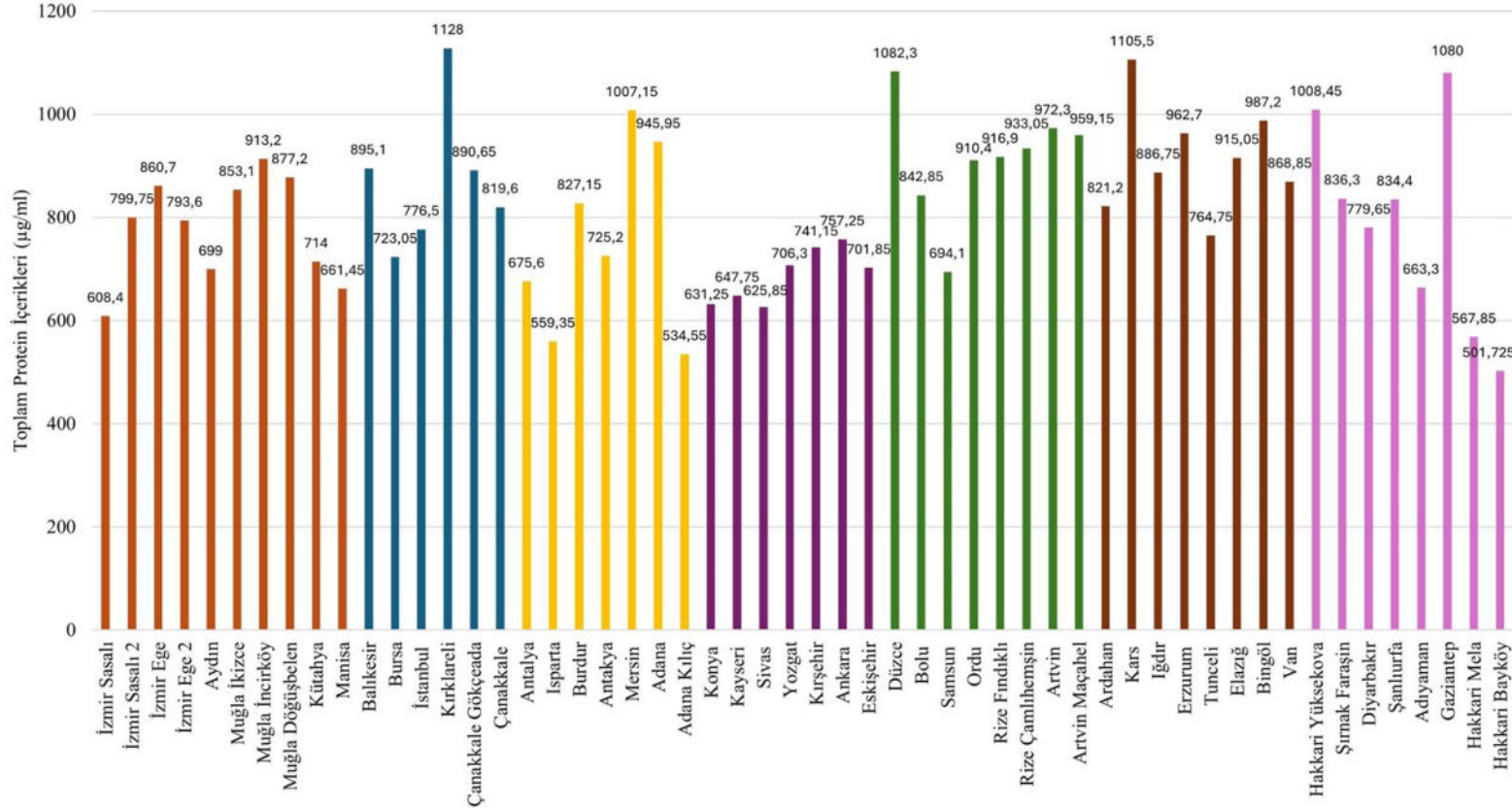
Statistical analysis: Collected bee venoms were statistically analyzed separately for protein content, cytotoxic activities and apoptosis test within geographical regions using one-way ANOVA test.



Work Flow of the Project



Results



- **BV Samples**

Our sample quantities range from **35.3 mg** to **303.3 mg** per hive.

- **Total Protein**

The protein concentration of *Apis mellifera* lyophilized crude venom (1 mg/ml) determined by the Smith assay was found between **559,35 µg/ml** and **1128 µg/ml** for samples.

The total protein content of the venoms was determined and classified geographically into seven regions. When evaluated on a regional basis, bee venom collected from the **Eastern Anatolia**, **Black Sea** and **Southeastern Anatolia** regions is found to have a higher **total protein content**.

Results

Venom Samples / Cell Lines	MDA-MB-231 (Breast)	PC-3 (Prostate)	U87MG (Glioblastoma)	A549 (Lung)	HeLa (Cervix)	PANC-1 (Pancreas)	Caco-2 (Colon)	CCD-34Lu (Healthy cells)
İzmir Sasalı 2	6.79 ± 0.76	3,36 ± 0,40	14.00 ± 1.43	5,59 ± 1,57	9,23 ± 0,03	5,18 ± 2,01	15.98 ± 1.28	12,88±2,60
Muğla İncirköy	16.73 ± 0.65	8,61 ± 1,97	17.49 ±0.83	12,4 ± 1,75	13,6 ± 1,68	4,25±0,64	22.36 ± 2.74	17,01 ±0,38
Kırklareli	12.75 ± 2.18	7,88 ± 1,66	26.37 ± 2.22	17,97 ± 1,95	10,76 ± 0,09	5,36± 0.31	19,91 ± 0,79	20.9 ± 1.67
Çanakkale Gökçeada	11.61 ± 3.10	18,31 ± 2,92	24.37 ± 1.31	20,14 ±0,55	13,49 ± 0,07	5.66± 1,40	20,62 ± 1,79	23.44 ±0.51
Düzce	11.26 ± 0.02	12,3 ±0,72	23,34 ± 4,15	19,08 ± 1,07	10,71± 0,07	6,15 ± 0,96	20,51 ±0,70	26.07±2.06
Rize Çamlıhemşin	11.53 ± 2.41	4,01 ± 0,63	14,49 ± 1,67	12,94 ± 2,76	8,13 ± 0,28	7,65 ± 0,19	10,13 ±1,55	16.91±1.64
Macahel	11.09 ± 0.34	5,23 ± 0,64	18.01 ± 0,74	10,16 ± 2,25	8,59 ± 0,06	8,28 ± 1,95	18,93 ±2,64	18.33±0.19
Hakkari Yüksekova	20,77 ± 2,89	7,65 ± 0,85	14.88 ± 0.68	16.46 ± 2.13	3.78 ± 0.09	7.05 ± 0.68	22,69± 0,53	17,22 ± 0,28
Şırnak Faraşın	18.55 ± 0.31	3,97 ± 0,68	16,59 ± 0,91	7.73 ± 1.77	14.06±1.76	7,52 ± 1,01	21.23 ±3.81	11,73±1,04
Adana	12,76 ± 2,58	6,54 ± 0,43	16.39 ± 0.40	19.68 ± 3.26	4.44 ± 0.30	4.59 ± 0.36	19,66 ± 1,08	17,89 ± 1,40
Hatay Antakya	13,24 ± 2,89	6,32 ± 0,35	15.29 ± 0.55	21.31 ± 1.17	3.95 ± 0.94	14.16± 2.00	20,95 ± 1,06	14,67 ± 0,19
Doxorubicin	14.87 ±3.32	3.35 ± 0,92	10.61 ±3.15	1,02 ± 0,51	4.00 ± 0,86	8.91 ±3.92	21.14 ± 0.60	6.12 ± 0,33

The most sensitive cell line in most regions was prostate adenocarcinoma (PC-3). In some regions, HeLa (cervical adenocarcinoma) and PANC-1 (pancreas) cells were also among the most sensitive. The most resistant cell lines were generally colorectal adenocarcinoma (Caco-2) and healthy lung cells (CCD-34Lu). IC50 values varied by region and bee venoms exhibited potent cytotoxic effects on cancer cells, although these effects differed by region. This table lists the most active venoms and the cancer cells on which they were most effective.

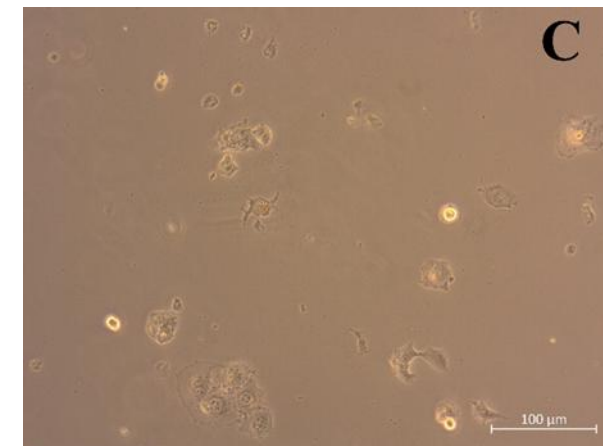


In vitro Cytotoxicity

- Colon Adenocarcinoma (Caco-2)
- Lung Carcinoma (A549)
- Breast Adenocarcinoma (MDA-MB-231)
- Cervical Adenocarcinoma (Hela)
- Glioblastoma (U87-MG)
- Prostate Adenocarcinoma (PC-3)
- Pancreatic Epithelial Carcinoma (PANC-1)
- Human Healthy Lung Cells (CCD-34Lu)

Results

According to the analysis results, the venoms of **Kırklareli (*Apis mellifera carnica*)**, **Gökçeada (*Apis mellifera anatoliaca* ecotype)**, and **Efe bee (*Apis mellifera anatoliaca* ecotype)** have anti-tumor effects on pancreatic epithelioid carcinoma (PANC-1), while **Maçahel (*Apis mellifera caucasia*)**, **Şırnak-Faraşın (*Apis mellifera anatoliaca* and *Apis mellifera meda* ecotype)**, **Cyprus Mallıdağ (*Apis mellifera cypria*)** bee venoms on human prostate adenocarcinoma (PC3), and **Adana and Antakya (*Apis mellifera syriaca* ecotype)** bee venoms on human cervical adenocarcinoma (HELA) cells.

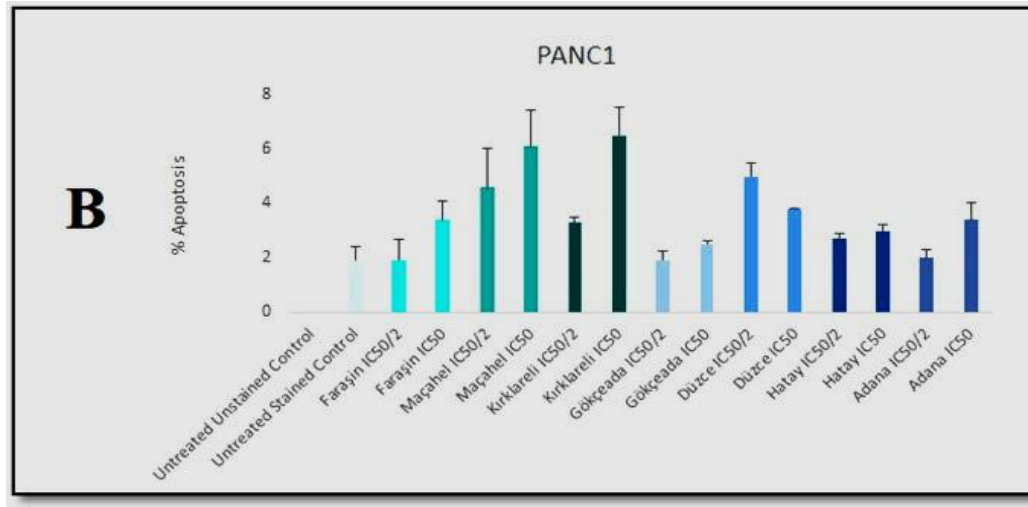
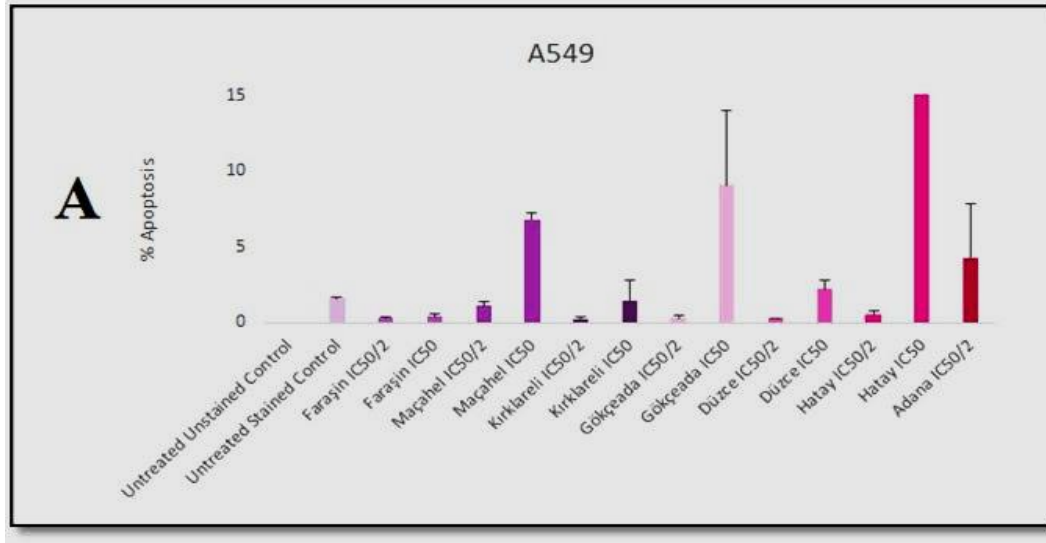


Dose-dependent morphologic changes of Panc-1 cells viewed by inverted microscope (10x).

A. Untreated (Control), **B.** Maçahel IC50/12 hours later after **C.** Maçahel IC50/24 hours later after

Results

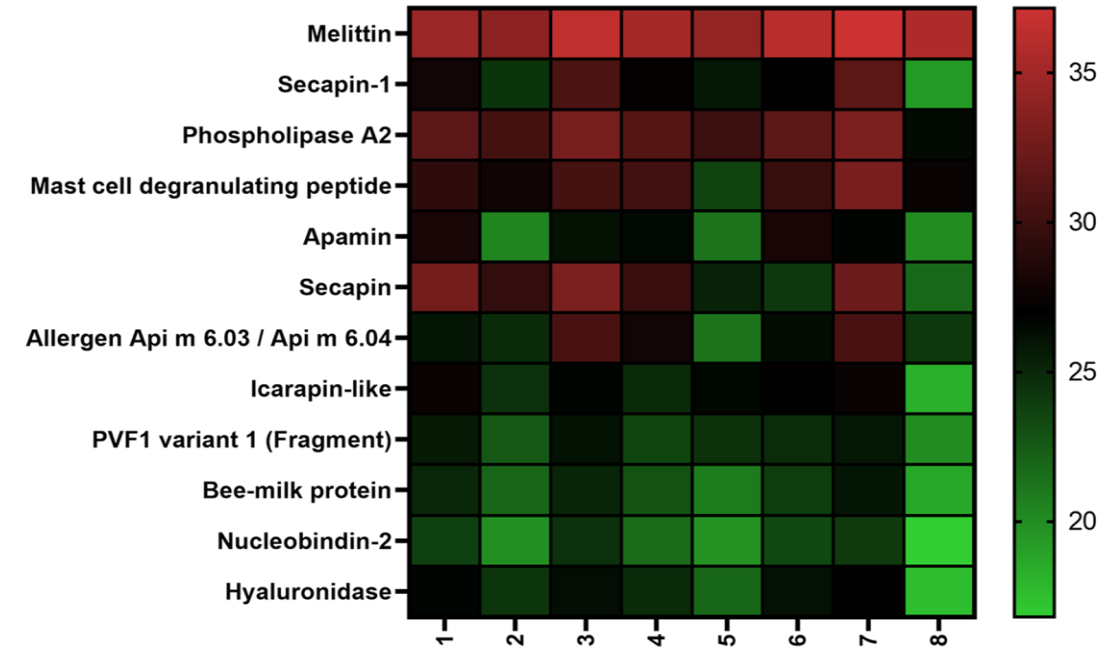
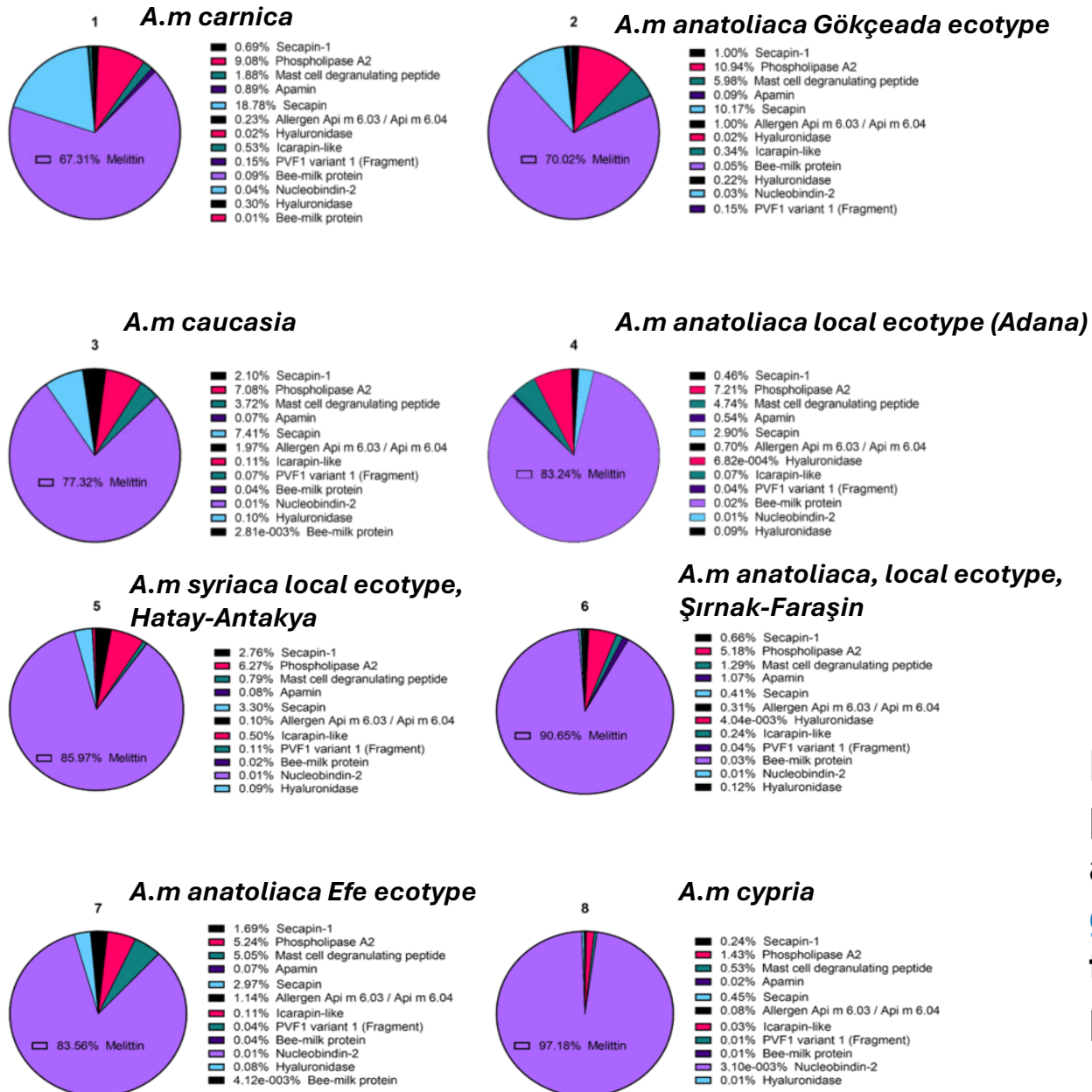
Apoptosis Tests



According to apoptosis test results, the regions with the highest apoptosis-inducing bee venom yields for the A549 (lung) cancer cell line were **Hatay, Gökçeada, Maçahel, and Adana.**

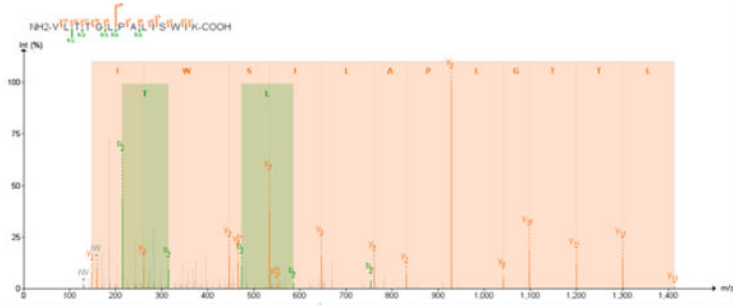
The regions with the highest apoptosis-inducing bee venom yields for the PANC1 (pancreas) cancer cell line were **Kırklareli, Maçahel, Düzce, and Şırnak-Farasın.**

Results – Proteomic analysis

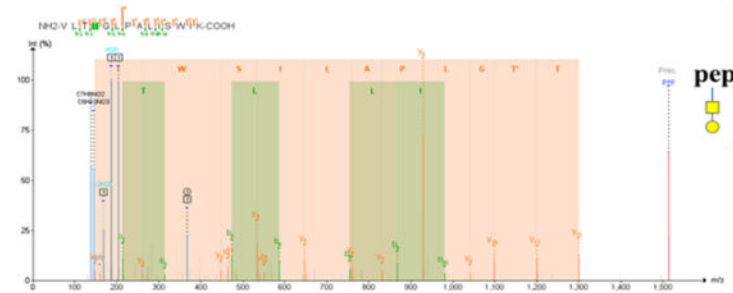


LS/MS analysis and genomic analyses were performed on samples collected from regions that are **biologically active, rich in protein content, and genetically significant**. The percentages given in these graphs represent the ratios within the total proteins in the venom content.

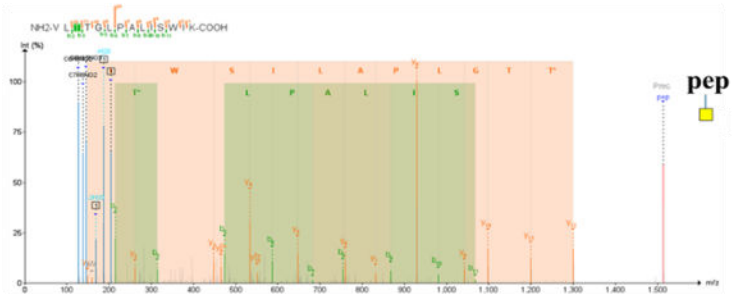
Results – Proteomic analysis



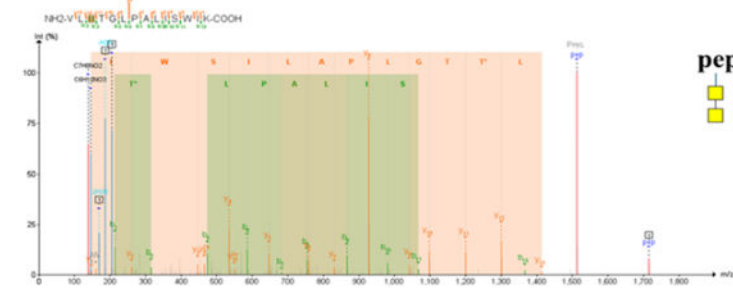
Peptide sequence of melitin without modification



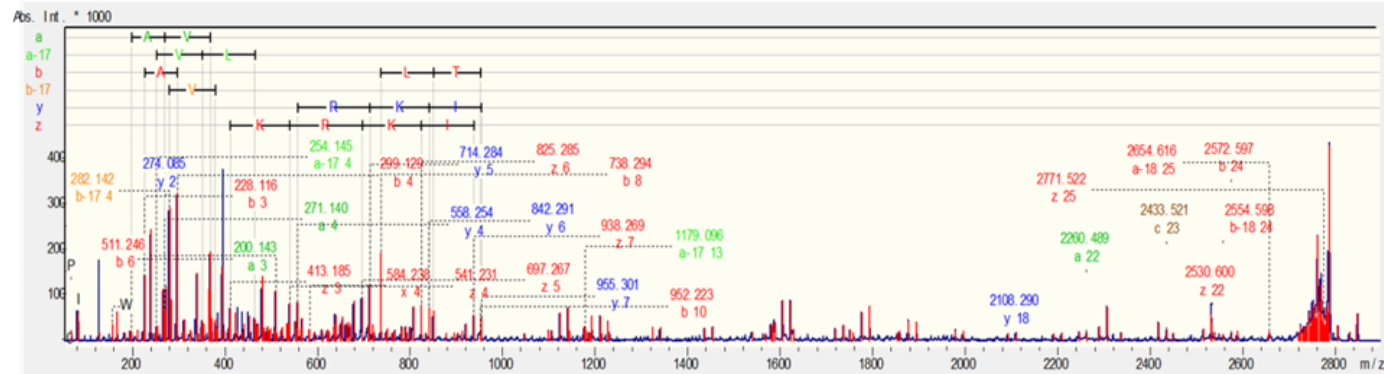
Melitin including O-glycan structure Hex1HexNAc1



Melitin including O-glycan structure HexNAc1



Melitin including O-glycan structure HexNAc2



The results revealed that melittin was O-glycosylated and appeared in varying abundances across species, which is significant for pharmaceutical applications.

Results – Genomic analysis

CLUSTAL 2.1 multiple sequence alignment

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1  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
2  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
3  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
4  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
5  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
6  MKFLVNVALVFMVVYIXYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
7  MKFLVNVALVFMVVYISYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
8  MKFLVNVALVFMVVYISYIYAAPEPEPEPEAEADAEADPEAGIGAVLKVLTTGLPALI
*****
  
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1  SWIKRKRQQG
2  SWIKRKRQQG
3  SWIKRKRQQG
4  SWIKRKRQQG
5  SWIKRKRQQG
6  SWIKRKRQQG
7  SWIKRKRQQG
8  SWIKRKRQQG
*****
  
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All samples, except for **Gökçeada** and **İzmir-Efe** exhibit a single identity. These locations indicate that a percentage of the bee colonies possess genetically variable lineages.

The discovery of structural variations in melittin holds high potential for pharmaceutical use and may lead to the development of new drug candidate proteins.

1. *A.m carnica*,
2. *A.m caucasica*,
3. *A.m. Anatoliaca* local ecotype (Antakya-Hatay)
4. *A.m anatoliaca* local ecotype (Adana)
5. *A.m syriaca* local ecotype
6. *A.m cypria*
7. *A.m anatoliaca* Gökçeada ecotype
8. *A.m anatoliaca* Efe ecotype



As a result

*The analysis of bee venoms obtained from different regions of the country and from different honeybee races and ecotypes shows that all collected venoms have dose-dependent cytotoxic and apoptosis-inducing effects on different cancer cells.

*According to the analysis results, *Kırklareli (Apis mellifera carnica)*, *Gökçeada (Apis mellifera anatoliaca ecotype)* and *Efe bee (Apis mellifera anatoliaca ecotype)* venoms were the most effective on pancreas epithelioid carcinoma (PANC-1); *Macahel (Apis mellifera caucasia)*, *Farasin (Local ecotype)*, *Kıbrıs Mallıdağ (Apis mellifera cypria)* venoms were found to be most effective on human prostate adenocarcinoma (PC3) while *Adana and Antakya (Apis mellifera syriaca local ecotype)* venoms appear to be most effective on human cervix adenocarcinoma (HELA) cells.

*Considering Turkey's phytogeographic diversity which affects the content and amount of bee venom, it is suggested that the country has an advantage in the field of venom research.



As a result

*Genomic and proteomic analysis results reveal that melittin occurs in varying abundances among species.

*Additionally, O-glycosylated forms of the melittin peptide were detected in all worked bee venoms, revealing multiple glycoforms.

*These findings provide deeper insights into the complexity of bee venom composition which could have important implications for both therapeutic applications and our understanding of venom variability.

*It is predicted that bee venoms obtained from pure honey bee species and ecotypes are more effective on cancer cell lines, that preservation of the local genotype may be an important factor in the planning of apitherapeutic bee venom production and that there is the potential to plan regional and controlled production of bee venom for different apitherapeutic purposes.

*In conclusion, bee venoms collected from Türkiye, appear to have high and diverse potential for pharmaceutical or apitherapeutic use. This high effect seen in the study results, supported by detailed scientific research, may be predicted as a valuable resource for the world bee venom industry and apitherapy practices.

Research Article

Ayşe Nalbantsoy*, Ekin Varol*, Ayşe Dila Çağlar and Banu Yücel

Cytotoxic and apoptotic effectiveness of Cypriot honeybee (*Apis mellifera cypria*) venom on various cancer cells

<https://doi.org/10.1515/tjb-2023-0109>

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Abstract

Objectives: The bee stinger is the defense organ of honeybees. The venom sac of a worker bee is connected to its stinger, which is used as a defense mechanism, and it has a potent and complex combination of substances that is unique in the animal kingdom. Many immune-related

showed that bee venom slightly induced early apoptosis on A549 and Panc-1 cells.

Conclusions: The venom of the *A. m. cypria* is discussed in this article, displaying promising results as a potential source for an alternative treatment method because of its cytotoxic effect.

Keywords: *Apis mellifera cypria*; cytotoxicity; apoptosis; Cypriot honeybee; venom



Cytotoxic and apoptotic potential of the different ecotypes of Anatolian Honeybee (*Apis mellifera* L.) Venoms on Various Cancer Cells (JAS-00241-2024-02), has been accepted for publication in Journal of Apicultural Science.





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Manavlar Company

And to you for your kind attendance