Original Article

CYTOTOXIC AND APOPTOTIC POTENTIAL OF DIFFERENT ECOTYPES OF ANATOLIAN HONEYBEE (*APIS MELLIFERA* L.) VENOMS ON VARIOUS CANCER CELLS

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Abstract

Honeybees play a crucial ecological and economic role by pollinating both natural ecosystems and cultivated crops. Beyond this, they produce such valuable natural products as honey, propolis, royal jelly, bee pollen, beeswax, and bee venom, which have been used for centuries in traditional medicine. With the growing interest in natural compounds for disease prevention and treatment, apitherapy and bee-derived products have gained renewed attention. Among these, bee venom (BV) stands out due to its rich composition of bioactive molecules, including peptides such as melittin, phospholipase A2 (PLA2), apamin, mast cell degranulation peptide, secapin, and adolapin, as well as enzymes as hyaluronidase. These components are known for their therapeutic potential, particularly in inflammation and cancer-related applications. In this study, the cytotoxic and apoptosis-inducing effects of *Apis mellifera* L. venom samples collected from fifty-one locations across Türkiye were tested against seven human cancer cell lines (Caco-2, PC3, U-87MG, MDA-MB-231, A549, HeLa, Panc-1) and one healthy line (CCD-34Lu). Cytotoxicity was assessed with use of the MTT assay, and IC_{50} values ranged from 1.64±0.70 to 98.33±9.28 µg/ml. Venoms from Maçahel, Faraşin, Kırklareli, Düzce, Adana, Hatay, and Gökçeada were the most potent, influenced by bee ecotypes and protein content. PC3, PANC-1, and HeLa were the most sensitive cell lines, while Caco-2 and CCD-34Lu showed the highest resistance. In conclusion, the diverse venoms of *A. mellifera* in Türkiye exhibit significant dose-dependent cytotoxic and apoptotic effects, positioning the country as a valuable source in venom-based therapeutic research.

Keywords: apoptosis, cytotoxicity, honeybee biodiversity, honeybee venom

INTRODUCTION

For many years, the European honeybee (*Apis mellifera* L.) has provided humans with a variety of therapeutic goods, including honey, propolis, and honeybee venom (Duffy et al., 2020). Among these products, bee venom (BV) stands as a rare and unique weapon, a powerful and complex substance that serves to protect bees from a wide range of predators, including vertebrates and other arthropods. According to numerous publications, BV contains a variety of bioactive molecules. This variety includes such peptides as melittin, mast cell degranulating peptide, apamin, secapin, adolapin, as well as proteins with enzymatic properties (phospholipase A2, hyaluronidase, α-d-glucosidase), and non-peptide components (dopamine, histamine, and norepinephrine) (Oršolić, 2012; Ceremuga et al., 2020). Additionally, a diverse range of low-molecular-mass substance, including sugars, amino acids, phospholipids, pheromones, and biogenic amines, are present in BV (Ceremuga et al., 2020).

Melittin and phospholipase A2 (PLA2) are the two principal bioactive components of bee venom (BV), with melittin alone constituting

approximately 50-60% of its dry weight. Melittin is unique to BV and is responsible for a wide range of biological effects, including potent cytotoxic, anti-inflammatory, and anticancer activities. However, it is also the most toxic component of BV (Park et al., 2011; Oršolić, 2012). Early studies, such as that by Hait et al. (1985), demonstrated melittin's ability to inhibit leukemic cell proliferation, a finding supported by later research exploring its anticancer potential (Liu et al., 2008; Jo et al., 2012). PLA2 is the second most abundant compound in BV and plays a dual role: it is both highly allergenic-sensitizing 57-97% of allergic individuals and therapeutically relevant due to its antimicrobial and immune-stimulating properties (Carpena et al., 2020; Yaacoub et al., 2021). Additionally, minor components of BV also contribute to its biological activity, offering neurotoxic, anti-inflammatory, anti-fibrinolytic, and antibacterial effects (Carpena et al., 2020).

Bee venom (BV) and bee-derived compounds have long been used in traditional medicine to treat chronic inflammatory conditions due to their anti-arthritic, analgesic, antioxidant, and anticancer properties (Khalil et al., 2021; El-Hanoun et al., 2020). Melittin, the main peptide in BV, exerts strong antimicrobial and antiviral effects by disrupting cell membranes and inhibiting the replication of viruses such as herpes simplex and murine retrovirus (Elieh Ali Komi et al., 2018; Carpena et al., 2020). Topical application of BV has also shown anti-inflammatory benefits, particularly in treating atopic dermatitis by reducing cytokine activity and IgE levels (Carpena et al., 2020). PLA2, another major component, has demonstrated neuroprotective effects through enhancing regulatory T cell (Treg) populations and has shown promise in Alzheimer's disease models (Ye et al., 2016). Moreover, BV has been associated with improved reproductive performance in animal models, likely due to its antioxidant capacity (El-Hanoun et al., 2020). In clinical settings, apitherapy, which utilizes BV through such methods as direct stings, injections, or acupuncture, has been applied to various conditions including neurological disorders, arthritis, musculoskeletal pain, and cancer (Lin et al., 2020). However, despite promising anticancer effects of melittin and PLA2, further research is needed to overcome challenges related to clinical safety, delivery systems, and large-scale trials (Cui et al., 2024).

These findings have contributed to growing interest in BV as a potential therapeutic agent, particularly in the field of oncology. However, the biological effects of BV can vary significantly depending on factors such as environmental conditions, bee genetics, and floral resources (Pucca et al., 2019; Maitip et al., 2021; Shi et al., 2022).

Türkiye, located at the intersection of Europe and Asia, harbors exceptional honeybee genetic diversity, including several native ecotypes of *Apis mellifera* L. that have adapted to distinct regional conditions (Kandemir et al., 2000; Kekeçoglu et al., 2020). However, limited studies have investigated how these ecotypic differences affect the therapeutic properties of BV, particularly in the context of cytotoxic and pro-apoptotic activities on cancer cells. Therefore, this study aims to evaluate the cytotoxic and pro-apoptotic potential of bee venom samples collected from different Anatolian honeybee ecotypes across various geographical regions of Türkiye. By assessing the effects of these venoms on selected cancer cell lines, we seek to deepen the understanding of how ecotype-specific biochemical differences may influence therapeutic efficacy, contributing to the development of more targeted and effective apitherapeutic applications.

MATERIAL AND METHODS

Bee venom

All tests were performed using collected venom of *A. mellifera* subspecies, collected from fifty-one apiaries from forty-three provinces across almost all of Türkiye (Fig. 1). Bee venom samples were collected with an electroshock device (Arsum Apimak Bee Venom Collection Machine, Ankara, Türkiye). The electroshock method is considered the safest method for collecting bee venom (de Graaf et al., 2021). The specifications of the bee venom collection machine are as follows: input voltage 12 VDC, and operating time 30 min.

Fig. 1. Representation of collected bee venom samples according to geographical regions on the map of Türkiye. One sample was collected from each location shown in red.

Sampling was conducted from the top of the hives between 12:00 pm and 15:00 pm, between May and August. Crude venoms were collected by scraping on the glass frame of the venom collector. The samples were stored in airtight amber bottles and a cooler bag until they reached the laboratory. After that, all samples were lyophilized, kept dry, and stored at +4°C in amber bottles. Before cell-based assays were performed, venom samples were diluted in physiological saline.

Protein content determination

Protein content was determined with bicinchoninic acid (BCA) assay (Pierce Kits, ThermoScientific, Bremen, Germany). The protein content of each diluted venom sample (1 mg/mL) was assessed described by the Smith (1985) method at a wavelength of 562 nm using an ultraviolet (UV)-visible spectrophotometer (Thermo, Bremen, Germany). The assay relies on the color change from green to purple. Colorimetric methods are used to determine the amount of protein in a solution since this change correlates directly with the total protein concentration.

Cell culture

The cytotoxic potential of the BV was demonstrated against human cancerous cells. In this study, human cervix adenocarcinoma (HeLa), human breast adenocarcinoma (MDA-MB-231), human alveolar carcinoma (A549), human glioblastoma-astrocytoma (U-87 MG), human colorectal adenocarcinoma (Caco-2), pancreas epithelioid carcinoma (PANC-1), human prostate adenocarcinoma (PC-3) and human healthy lung cells (CCD-34Lu) were used. All the cell lines used for the experiment were purchased from ATCC (Manassas, VA, USA). Cells are maintained in Dulbecco's modified Eagle's medium F12 (DMEM/F12), containing 10% heat-inactivated fetal bovine serum (FBS), 0,1% penicillin/ streptomycin. The cell growth process was performed in a humidified incubator at 37°C and 5% CO_2. The cells were subcultured twice a week.

***In vitro* cytotoxicity assay**

A modified colorimetric MTT [3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide)] test is used to determine the cytotoxicity of the crude venom (Mosmann, 1983). In the MTT assay, the amount of viable cells were to be determined through the measurement of mitochondrial reductase activity. MTT, a tetrazolium salt, is transformed into formazan crystals, which are used to measure mitochondrial activity. Through the measurement of the optical density (OD) of

the wells in the 96-well plates, the increase or decrease of the viable cells is detected (van Meerloo et al., 2011).

All samples collected from forty-three provinces and fifty-one apiaries in Türkiye were treated with seven cancerous cell lines and one healthy cell line at determined concentrations. BVs were dissolved in physiological saline to obtain a stock solution of 10 mg/ml. To perform the analysis, all cell lines were cultivated in 96-well microplates with an initial concentration of 10^5 cells/ml for 24 h. Next, cells were treated with three different concentrations of 50, 5, and 0,5 µg/ml of venom samples diluted in physiological saline and incubated for 48 hours at 37°C. As a positive control, Doxorubicin was used in three different concentrations. Following the incubation, MTT solution (1 mg/mL) is added to all wells, and the plates are incubated for a further four hours at 37°C. After the four hours, the MTT solution was discarded and the formed formazan crystals were dissolved in 150 µL DMSO. At a wavelength of 570 nm, the amount of produced formazan crystals was determined calorimetrically through UV-visible spectrophotometry (Thermo, Bremen, Germany). The cell viability (%) is determined using the formula:

$$\%\ \text{Viability} = \frac{(absorbance\ of\ treated\ cells) - (absorbance\ of\ blank)}{(absorbance\ of\ control) - (absorbance\ of\ blank)} * 100$$

Determination of IC_{50}

OD measurements were used to calculate the half-maximal inhibitory concentration (IC_{50}), which is the concentration of venom that inhibits cell growth by 50% compared to untreated controls. Cytotoxicity was calculated according to a decrease in the mean percentage of cell viability relative to the unexposed control ±SD. Cell viabilities of the control groups were accepted as 100%. Calculations were performed with the use of GraphPad Prism 9 software (GraphPad9, San Diego, CA, USA), and the IC_{50} values were reported at a 95% confidence interval.

Flow-cytometry analysis for apoptosis

The apoptosis test was performed to analyze the mechanisms of anti-cancer effects of bee venoms selected from the most suitable samples based on protein content and cytotoxic activity. Through the use of 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. Since lung and pancreas cancers are among the most common and most lethal cancers in the world, A549 and PANC-1 cell lines were selected. A549 and PANC-1 cell lines were seeded ata density of 5×10^5 cells/well and $7{,}5\times10^4$ cells/well, respectively, and cultured for 24 h at 37°C with 5% CO_2. Cells were then exposed to the determined IC_{50} and $IC_{50}/2$ concentrations of the selected bee venoms. A549 cells were incubated for 24 hours, and PANC-1 cells were incubated for 48 hours, which were the optimum incubation periods determined by preliminary studies. Following the incubation period, cells are harvested using 1X trypsin and collected in 2ml Eppendorf tubes to wash twice with cold PBS through the use of centrifugation at 1000 rpm and 4°C for 5 min. Then, the cells were suspended in 100 µl of 1X Annexin V binding buffer. Further analysis was performed with the use of the FITC-Annexin V apoptosis detection kit I (BD Pharmingen; BD Biosciences), according to the protocol described by the manufacturer. Apoptosis ratios in the experimental groups were analyzed by gating untreated cells stained with Annexin V on the FL-1 plot. Samples, each containing 10^4 cells, were then analyzed with the use of a flow cytometer (BD Accuri™ C6 instrument; BD Biosciences) and BD Accuri™ C6 software (version 1.0.264.21; BD Biosciences).

SDS - PAGE Analysis

SDS-PAGE was used to control the purity of the complex combination of bee venom proteins through their separation according to their molecular weight. 15% resolving gel (250 mM Tris/HCl pH 6.8, 2.50 mL PAA, 50 µL SDS, 1.17 mL ultrapure water, 25 µL ammonium persulfate, 10 µL TEMED) and 4% stacking gel for SDS-PAGE gel (15.1 g Tris/HCl pH 6.8, 0.34 mL PAA, 25 µL SDS, 1.50 mL ultrapure water, 12.5 µL ammonium persulfate, 10 µL TEMED) were utilized.

The samples were separated into eight groups prior to analysis, loaded with Laemmli buffer, and the separated proteins in the gel were stained with Coomassie blue to enable sample visualization (Nalbantsoy et al., 2024).

Statistical analysis

Statistical analysis was conducted with the use of two software programs: SPSS for Windows 10.0 and GraphPad Prism 8.4.2. The collected BVs were divided into seven geographical regions, and their protein contents were statistically analyzed within these regions with the use of a one-way ANOVA test, separately. The cytotoxic activities of BVs were also statistically analyzed with the use of the one-way ANOVA test based on these locations and compared with the doxorubicin activities. Furthermore, the statistical significance between the protein content of the samples and their cytotoxic activity on different cell lines was also determined through the use of hierarchical cluster analysis (HCA) based on a Squared Euclidean distance calculation and Ward's method. The results were compared according to the regions where the bee venoms were collected and also among the cell lines separately. The obtained dendrograms were expressed as heat maps. The apoptosis assay results were compared with the control group using the one-way ANOVA test and adjusted using Dunnett's analysis. Values were expressed as the mean ±SD, and the value of $p<0.05$ was considered statistically significant.

RESULTS

Protein content determination

The protein concentration of *A. mellifera* lyophilized crude venom (1 mg/ml) was determined by the Smith assay to be between 1128 µg/ml and 559.35 µg/ml for samples collected from forty-three provinces of Türkiye, as shown in Table 1. There was no significant difference between the statistically analysed BVs within the geographical regions where they were collected.

***In vitro* cytotoxic activity**

Cytotoxicity assays were performed on various human cancerous cell lines(MDA-MB-231, U-87 MG, A549, PC-3, HeLa, PANC-1, and Caco-2) and one healthy cell line (CCD-34Lu) with crude venom of *Apis mellifera* L. collected from forty-three provinces almost all over Türkiye. The venom's effect on cells was evaluated via the MTT assay after treatment with different concentrations of crude venom for 48 hours.

IC_{50} values of the cells treated with venom collected from the Aegean Region varied between 3.36±0.40 µg/ml and 25.71±0.33 µg/ml (Tab. 2). Among all cell lines, the most potent activity was demonstrated against the prostate adenocarcinoma cell line (PC-3) and the pancreas epithelioid carcinoma (PANC-1), while the most resistant cell line was colorectal adenocarcinoma (Caco-2). The IC_{50} values of the cells treated with venoms collected from the Marmara Region were found to be between 3.81±1.30 µg/ml and 27.75±4.32 µg/ml (Tab. 3). The prostate adenocarcinoma cell line (PC-3) was the most sensitive cell line among the cell lines, while the human glioblastoma-astrocytoma (U-87 MG) cell line was found to be the most resistant. Venom collected from the Central Anatolia Region were most potent against the PC-3 cell line with the lowest IC_{50} value of 1.64±0.70 µg/ml, and the most resistant cell line was the human healthy lung cells (CCD-34Lu) with the highest IC_{50} value of 28.9±1.58 µg/ml (Tab. 4). The venom of the bees from the Black Sea Region was found to be most effective against the PC-3 cell line, and the most resistant cell line was CCD-34Lu (Tab. 5). The IC_{50} values of the cells varied between 3.81±1.13 µg/ml and 26.07±2.06 µg/ml. The venom collected from the Eastern Anatolia Region were the most effective on the human cervix adenocarcinoma (HeLa) cell line (Tab. 6). The lowest IC_{50} value belonged to the HeLa cells treated with the venom from Erzurum Palandöken with a value of 3.26±0.54 µg/ml, while the highest value was calculated as 26.31±0.11 µg/ml for human alveolar carcinoma (A549) cells. The PC-3 cells were found to be the most sensitive cell line amongst the treated cell lines,

Table 1.

Protein concentrations of the venoms collected from different regions across Türkiye (µg/ml)

Venom Collection Area	Protein Concentration (µg/ml)
Aegean Region	
İzmir Sasalı	608.40±12.20 ns
Muğla İncirköy	913.20±9.10 ns
Aydın Kosk	699.00±15.5 ns
Muğla İkizce	853.10±10.20 ns
Muğla Doğuşbelen	877.20±8.80 ns
Kütahya	714.00±17.90
İzmir Ege	860.70±16.20
Manisa	661.45±6.60
İzmir Ege 2	793.60±15.80
İzmir Sasalı 2	799.75±9.60
Marmara Region	
Balıkesir	895.10±9.00
Bursa	723.05±18.10
İstanbul Çatalca	776.50±15.50
Kırklareli	1128.00±11.30
Çanakkale Gökçeada	890.65±20.30
Çanakkale Bayramiç	819.60±9.80
Central Anatolia Region	
Konya Abditolu	631.25±10.10
Kayseri Tomarza	647.75±13.00
Sivas Onbaşılar	625.85±6.30
Yozgat	706.30±17.70
Kırşehir	741.15±8.90
Ankara Kızılcahamam	757.25±7.60
Eskişehir	701.85±14.50
Blacksea Region	
Düzce	1082.30±27.10
Bolu Abant	842.85±10.10
Samsun Bafra	694.10±13.90
Ordu Perşembe	910.40±9.10
Rize Fındıklı	916.90±22.90
Rize Çamlıhemşin	933.05±11.20
Artvin Merkez	972.30±19.40
Maçahel	959.15±9.60
Eastern Anatolia Region	
Ardahan	821.20±16.40
Kars Kağızman	1105.50±11.10
Iğdır	886.75±22.20
Erzurum Palandöken	962.70±11.60

Tunceli Ovacık	764.75±19.10
Elazığ Sivrice	915.05±11.00
Bingöl	987.20±9.90
Van Çatak	868.85±17.40
Hakkari Yüksekova	1008.45±10.10
South Eastern Anatolia Region	
Şırnak Farasin	836.30±16.70
Diyarbakır	779.65±7.80
Şanlıurfa	834.40±20.90
Adıyaman	663.30±8.00
Gaziantep	1080.00±27.10
Mediterranean Region	
Antalya Elmalı	675.60±13.50
Isparta	559.35±5.60
Burdur	827.15±16.50
Hatay Antakya	725.20±7.30
Mersin Tarsus	1007.15± 3.20
Adana	945.95±11.40

ns: $p > 0.05$

with an IC_{50} value of 3.97±0.68 µg/ml. The most resistant cell line against the venoms were the Caco-2 and CCD-34Lu cell lines (Tab. 7). The venoms collected from the Mediterranean region showed the most potent effect on the HeLa cell line, with the lowest IC_{50} value of 3.42±0.07 µg/ml (Tab. 8). The Caco-2 cell line was the most resistant, with the highest IC_{50} value of 25.22±2.31 µg/ml.

Evaluation of the cytotoxicity results considering the protein contents showed that the samples collected from seven different geographical regions of Türkiye were regionally close to one another, although there were differences and exceptions for some samples. IC_{50} values and protein contents were statistically evaluated by hierarchical cluster analysis (HCA) and interpreted in terms of the regions where the samples were collected and cancer cell lines separately (Fig. 2). Cytotoxic activity varied according to geographical regions. According to hierarchical cluster analysis, samples with high protein content showed statistically significant results in terms of cytotoxic activity against PANC-1 and PC-3 cells. In addition, IC_{50} values of A549 and MDA-MB-231 cells, and IC_{50} values of Caco-2 and U87MG cells showed statistically similar results. The samples were divided into five groups based on the locations where they were collected. Among these, Artvin-Merkez, Erzurum-Palandöken, Adana, Bingöl, which have higher protein content, provided significant results by providing samples with similar IC_{50} profile.

The highest IC_{50} value (50 µg/ml) is shown in dark blue and the lowest IC_{50} value in white (50 µg/ml). Similarly, The highest protein content (1250 µg/ml) is shown in dark blue and the lowest protein content in white (0 µg/ml).

Based to HCA, samples collected from the different areas were categorized into five main classes and sorted from top (group 1) to bottom (group 5) on the statistical graph. While the group, including Adana and Artvin Maçahel samples, exhibited significant results in terms of cytotoxic activity, these samples also had higher protein content after the 4th group, including Düzce and Kırklareli samples. The cytotoxic activity of the samples in the 4th group was not as high as of the 5th group. Group 3, which includes Şırnak Farasin and Çanakkale Gökçeada, indicated average results in terms of protein content compared to other regions, while IC_{50} values show remarkable results. Samples collected from

Table 2.

IC_{50} values for *Apis mellifera* L. venom collected from Aegean Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
İzmir Sasalı	8.52±2.28	5.68±0.51	14.63±0.89	5.65±0.44	7.81±0.06	3.95±1.15	19.09±2.99	10.02±2.54
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
Aydın Köşk	12.56±2.18	8.07±2.50	17.07±0.78	9.63±1.50	15.13±2.05	4.31±0.53	21.45±2.08	15.54±0.60
Muğla İkizce	12.94±0.43	6.94±1.79	18.00±0.52	11.71±2.51	13.24±1.91	5.17±1.81	22.50±1.83	12.07±1.27
Muğla Doğuşbelen	20.76±2.60	8.28±1.39	17.82±0.14	14.84±1.00	15.05±1.96	7.57±0.03	25.71±0.33	19.12±0.96
Kütahya	17.71±0.45	10.23±0.19	17.18±0.36	13.04±0.70	18.81±0.44	5.29±1.96	22.16±1.58	17.03±0.34
İzmir Ege	16.65±0.15	8.61±0.94	18.33±0.47	16.53±1.09	17.48±2.24	6.89±2.28	20.47±3.42	15.51±0.03
Manisa	13.23±0.47	6.33±0.81	18.79±2.61	13.88±1.54	10.05±0.21	6.65±1.36	19.23±0.44	19.47±1.31
İzmir Ege 2	13.05±0.69	3.79±1.23	17.68±2.51	4.74±1.44	11.82±1.98	5.19±1.17	22.90±0.54	15.66±0.65
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
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

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

Table 3.

IC_{50} values for *Apis mellifera* L. venom collected from Marmara Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Balıkesir	14.34±0.33	6.78±0.02	26.97±3.47	18.37±1.42	9.16±0.13	7.22±0.90	18.16±0.69	19.46±0.05
Bursa	15.04±0.41	7.12±1.18	25.37±2.74	18.67±2.40	9.28±0.30	9.46±1.65	15.62±1.61	19.92±2.06
İstanbul Çatalca	10.06±0.56	6.69±2.28	27.75±4.32	17.53±1.47	10.52±0.77	7.09±1.71	22.27±1.45	25.54±1.35
Kırklareli	12.75±2.18	7.88±1.66	26.37±2.22	16.29±0.42	10.76±0.09	5.36±0.31	19.91±0.79	20.90±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
Çanakkale Bayramiç	12.39±0.73	4.32±0.31	22.38±0.62	11.86±1.31	7.36±0.00	3.81±1.30	16.88±0.90	22.87±1.61
Doxorubicin*	12.42±0.33	4.62±0.88	7.04±1.88	18.37±1.42	1.92±0.41	6.15±0.96	17.58±2.82	6.32±0.03

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

Groups 1 and 2 are close to each other in protein content (those with the lowest content) and cytotoxic activity. The samples collected from the Central Anatolia region had similar results to those collected from the Aegean region, while the samples collected from the Black Sea and Eastern Anatolia regions showed statistically identical responses. In addition, samples collected from the Mediterranean, Marmara, and South Eastern Anatolia regions displayed a wider distribution and demonstrated statistically close results with the other areas.

Table 4.

IC_{50} values for *Apis mellifera* L. venom collected from Central Anatolia Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Konya Abditolu	19.3±5.52	1.64±0.7	22.01±0.29	4.39±0.09	11.67±2.56	9.16±1.68	15.31±3.16	26.61±2.55
Kayseri Tomarza	16.47±1.46	3.16±1.04	22.38±1.05	7.67±0.60	13.21±2.49	10.35±1.13	19.72±2.41	24.51±0.81
Sivas Onbaşılar	12.49±1.21	4.14±0.86	14.33±0.43	7.54±0.85	21.89±0.85	8.85±0.47	30.92±1.37	21.72±2.76
Yozgat	18.47±2.17	3.98±0.97	21.83±0.21	8.22±0.12	12.04±0.88	7.67±1.48	18.99±1.39	26.92±2.55
Kırşehir	19.57±2.19	4.88±2.10	22.16±0.63	5.97±1.95	12.56±1.93	10.39±0.35	21.02±2.59	27.5±0.36
Ankara Kızılcahamam	12.17±1.09	1.66±0.69	21.5±0.14	7.38±2.34	7.98±1.94	12.44±3.09	17.85±2.22	28.9±1.58
Eskişehir	12.05±0.62	9.38±1.99	21.61±0.24	13.28±2.20	6.90±0.21	7.78±1.99	22.18±2.58	22.8±0.38
Doxorubicin*	17.22±1.66	5.24±0.44	5.70±0.94	1.02±0.51	4.36±0.26	2.95±0.04	19.71±2.63	6.33±0.02

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

Table 5.

IC_{50} values for *Apis mellifera* L. venom collected from Blacksea Region on cell lines following crude venom exposure (µg/ml).

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Düzce	11.26±0.02	12.33±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
Bolu Abant	18.45±0.06	9.57±2.88	15.94±3.51	18.21±1.84	10.25±0.92	3.81±1.13	22.65±2.83	23.13±0.03
Samsun Bafra	11.52±1.11	5.72±0.46	16.07±0.19	13.43±2.48	7.98±1.23	8.70±1.33	11.42±0.75	22.87±2.12
Ordu Perşembe	11.98±0.76	4.67±0.88	13.00±1.08	12.45±2.42	8.16±0.94	10.22±1.46	13.77±1.99	18.38±0.86
Rize Fındıklı	10.9 ±0.62	4.57±1.58	8.29±1.39	14.05±0.57	8.38±1.35	11.95±0.90	12.34±0.93	21.76±1.06
Rize Çamlıhemşin	11.53±2.41	4.01±0.63	14.495±1.67	12.94±2.76	8.13±0.28	7.65±0.19	10.13±1.55	16.91±1.64
Artvin Merkez	14.39±0.68	4.55±1.29	17.43±1.83	13.69±1.60	8.85±1.31	10.04±1.33	11.34±1.93	13.76±0.19
Artvin 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
Doxorubicin*	12.32±0.15	4.00±0.87	11.01±1.52	2.11±0.77	1.93±0.41	2.90±0.04	17.58±2.82	6.30±0.03

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

Apoptosis assay

Apoptosis assays of the bee venoms selected according to the results of protein content and cytotoxic activity and representing the best results in different regions were performed, and the results were compared with the control group through One-Way ANOVA using Dunnett's analysis. After the cells were stained with annexin V-FITC, an apoptosis detection kit was used to assess the apoptotic and cell death rate. Untreated cells were used as the control group. Results showed that the Izmir Sasalı, Artvin Macahel and Sırnak Farasin venoms had a dose-dependent early apoptosis-inducing

Table 6.

IC_{50} values for *Apis mellifera* L. venom collected from Eastern Anatolia Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Ardahan	19.54±2.85	8.78±0.39	17.35±1.88	16.48±1.81	5.38±1.22	5.99±1.22	20.35±0.02	15.94±3.51
Kars Kağızman	19.93±1.23	12.04±2.96	21.26±0.71	19.03±1.73	6.57±0.01	9.67±1.39	22.67±1.38	16.62±0.55
Iğdır	12.73±1.77	7.70±1.38	19.49±1.01	18.59±0.42	5.86±1.07	6.06±0.32	21.93±1.38	14.45±0.61
Erzurum Palandöken	10.27±0.89	7.75±0.39	19.53±1.24	16.99±0.54	3.26±0.54	5.85±0.42	21.96±0.12	15.31±0.76
Tunceli Ovacık	9.49±2.07	7.78±1.61	19.07±0.82	8.61±1.74	3.70±0.41	4.85±0.86	21.71±1.54	11.77± 1.60
Elazığ Sivrice	14.33±1.83	6.74±1.38	20.14±1.73	12.12±0.12	4.9±0.99	9.85±1.91	21.76±0.03	13.42±2.49
Bingöl	11.44±0.88	10.67±1.91	20.05±1.51	17.36±1.97	6.70±1.42	6.73±0.54	17.69±1.95	16.50±0.35
Van Çatak	16.12±2.09	9.10±9.25	14.17±0.27	26.31±0.11	4.77±0.05	6.62±1.86	21.87±0.38	18.16±0.59
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
Doxorubicin*	15.22±1.98	7.04±2.14	1.37±3.06	11.27±0.01	3.15±0.86	6.14±1.98	15.59±1.41	8.01± 1.33

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

Table 7.

IC_{50} values for *Apis mellifera* L. venom collected from South Eastern Anatolia Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Şırnak Farasin	18.55±0.31	3.97±0.68	16.59±0.91	7.73±1.77	13.12±0.17	7.52±1.01	21.23±3.81	11.73±1.04
Diyarbakır	17.56±0.79	6.93±1.12	23.34±3.93	20.47±0.78	14.44±2.56	6.67±1.52	20.64±1.73	18.19±0.02
Şanlıurfa	13.78±2.48	6.86±1.33	17.81±0.93	15.35±3.28	14.63±0.81	5.85±0.71	18.74±2.78	>50
Adıyaman	17.37±0.16	5.66±0.22	20.27±0.43	14.07±0.48	12.33±0.28	5.38±0.84	20.43±0.83	19.81±0.74
Gaziantep	17.78±0.37	7.69±0.80	19.93±2.61	13.91±2.81	13.91±2.81	7.90±0.07	16.03±1.60	18.16±1.55
Doxorubicin*	12.53±3.31	2.71±1.35	8.38±1.55	3.57±0.51	13.16±6.21	11.69±3.92	20.72±0.60	5.88±0.33

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

effect on the tested cell lines (Fig. 3). IC_{50} and $IC_{50}/2$ doses of the bee venom sample collected from Maçahel induced apoptosis in both A549 and PANC-1 cells in the early apoptosis phase with a higher level of significance ($p<0.0001$). In addition, the IC_{50} dose of bee venom sample collected from Faraşin was also effective on the A549 cell line in the early apoptosis phase ($p<0.0001$). On the contrary, no statistical difference was detected in the late apoptotic phase and necrosis assays in any bee venom compared to the control group ($p>0.05$).

SDS-PAGE analysis

As a result of SDS-PAGE analysis, five to fourteen protein bands were displayed on the gels, respectively. A dense protein band with a molecular weight of less than 5 kDa represents

Table 8.

IC_{50} values for *Apis mellifera* L. venom collected from Mediterranean Region on cell lines following crude venom exposure (µg/ml)

Venom Samples	MDA-MB-231	PC-3	U87MG	A549	HeLa	PANC-1	Caco-2	CCD-34Lu
Antalya Elmalı	25.22±2.31	10.96±2.73	15.86±1.62	24.13±2.74	4.24±0.39	13.25±2.40	23.13±0.60	14.77±0.14
Isparta	20.30±0.83	11.04±0.93	16.85±0.67	24.26±1.75	4.98±0.32	14.93±2.22	22.10±0.33	14.96±0.08
Burdur	19.11±0.05	11.28±2.83	15.79±1.74	16.35±0.43	3.42±0.07	15.17±1.77	21.27±0.35	15.50±0.06
Hatay Antakya	13.24±2.89	6.32±0.35	15.29±0.55	21.31±1.17	3.95±0.94	14.63±1.97	20.95±1.06	14.67±0.19
Mersin Tarsus	20.34±1.38	8.41±1.06	15.70±0.13	21.03±1.28	4.00±0.79	12.53±1.28	23.07±0.57	18.32±0.55
Adana	12.76±2.58	6.54±0.43	16.39±0.40	19.78±3.41	4.44±0.30	4.59±0.36	19.66±1.08	17.89±1.40
Doxorubicin*	15.22±1.97	25.48±0.01	1.37±3.06	11.27±0.01	4.00±0.86	6.14±1.91	15.59±1.40	8.01±1.33

*Cytotoxic agent as positive control. All data were expressed as the mean ±SD.

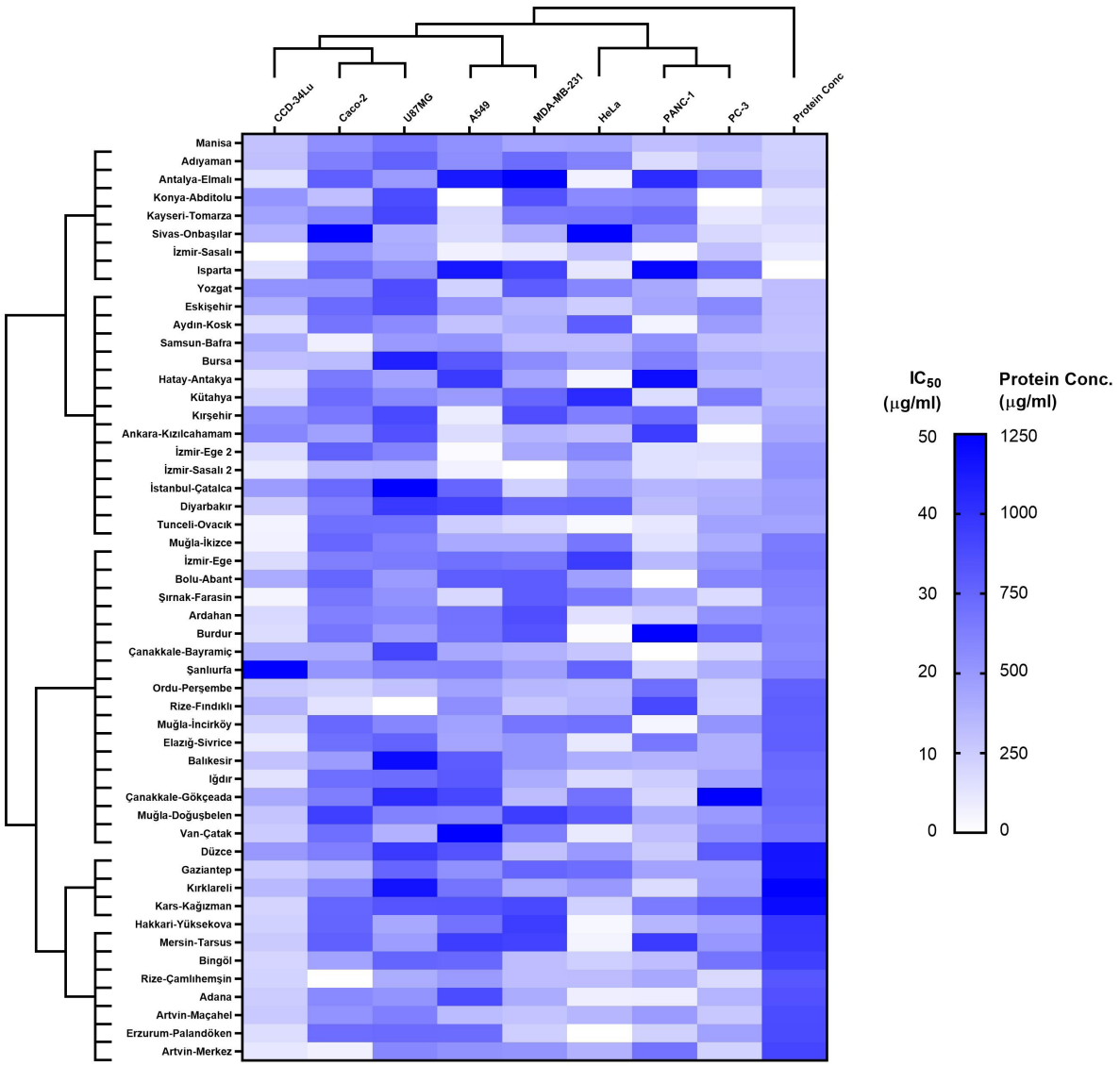

Fig. 2. Statistical comparison of BV samples collected from fifty-one different locations in forty-three provinces, calculated by hierarchical cluster analysis (HCA) based on their protein content and cytotoxic activity against different cell lines.

the intense protein band in all samples. Due to its low molecular weight, melittin (MEL) was seen to be the protein that traveled the furthest. Besides, that two other important protein bands determined to display clearly in the 20-40 kDa range were hyaluronidase (HLA) and phospholipase A2 (PLA) enzymes, respectively (Figs. 4, 5, 6, 7, 8, 9, 10 and 11).

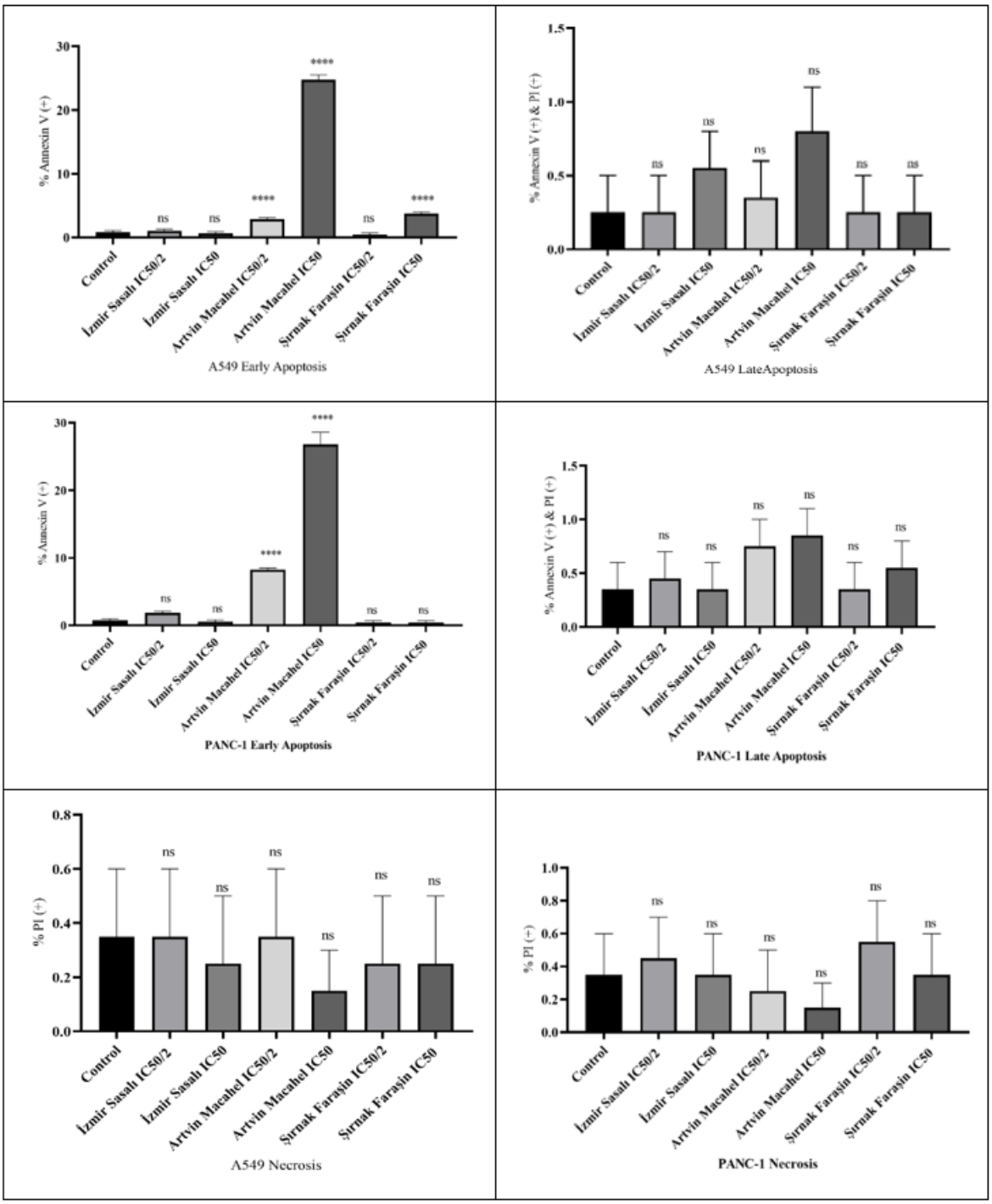

Fig. 3. Percentages of early apoptosis and late apoptosis as a result of Annexin V staining of Izmir Sasalı, Artvin Macahel, and Şırnak Faresin samples selected among BV samples.
Cells were exposed to IC50 and IC50/2 concentrations of these selected BVs. Untreated cells stained with Annexin V were gated in FL-1 graph to analyse the apoptosis rate of the experimental groups. Results are presented as mean value and standard deviation (SD).
ns: p>0.05, *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001.

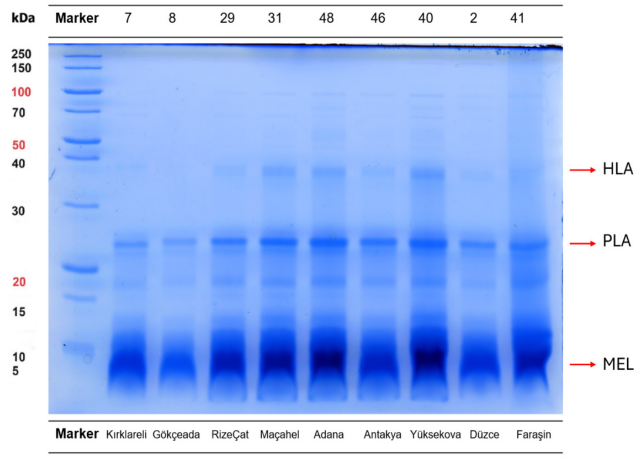

Fig. 4. SDS-PAGE gel imaging of proteins in bee venom for Group 1. Reference bands at 20, 50, and 100 kDa are indicated in red.

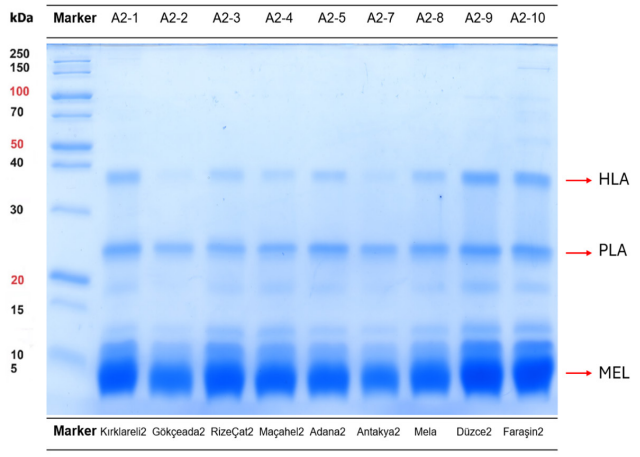

Fig. 5. SDS-PAGE gel imaging of proteins in bee venom for Group 2. Reference bands at 20, 50, and 100 kDa are indicated in red.

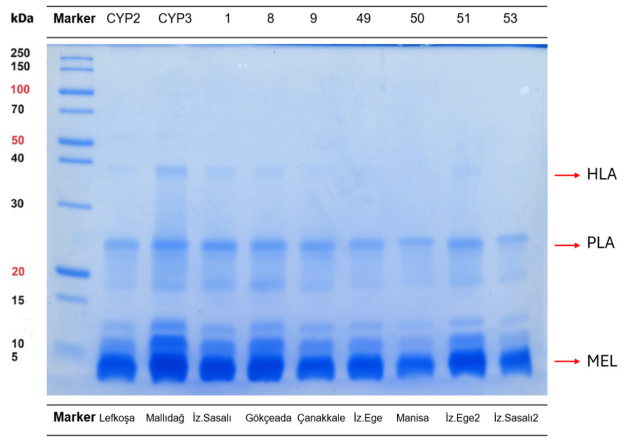

Fig. 6. SDS-PAGE gel imaging of proteins in bee venom for Group 3. Reference bands at 20, 50, and 100 kDa are indicated in red.

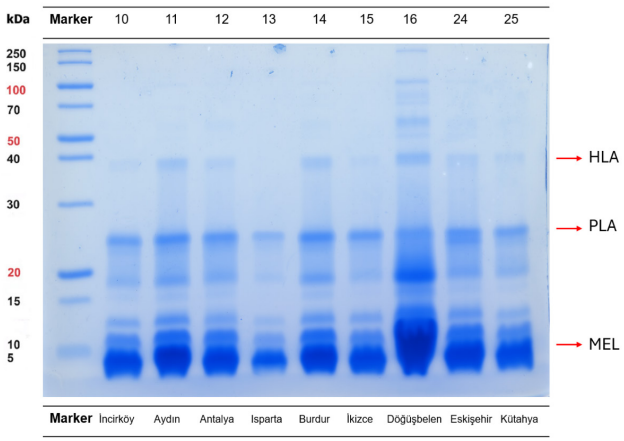

Fig. 7. SDS-PAGE gel imaging of proteins in bee venom for Group 4. Reference bands at 20, 50, and 100 kDa are indicated in red.

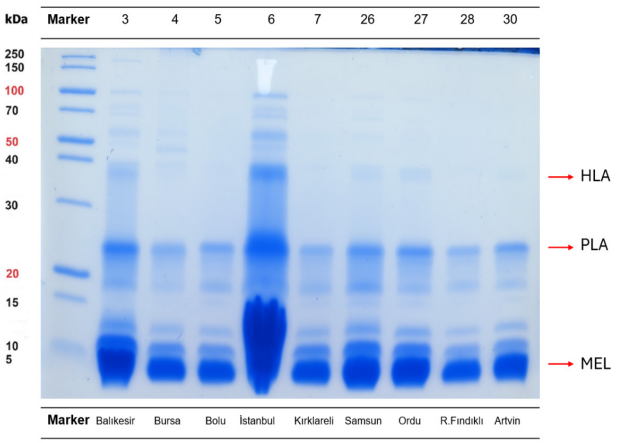

Fig. 8. SDS-PAGE gel imaging of proteins in bee venom for Group 5. Reference bands at 20, 50, and 100 kDa are indicated in red.

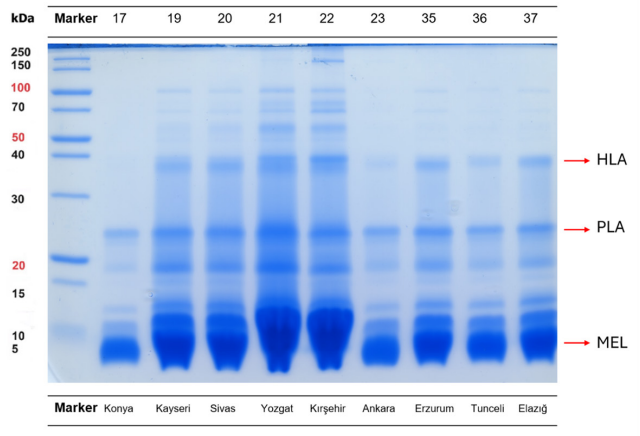

Fig. 9. SDS-PAGE gel imaging of proteins in bee venom for Group 6. Reference bands at 20, 50, and 100 kDa are indicated in red.

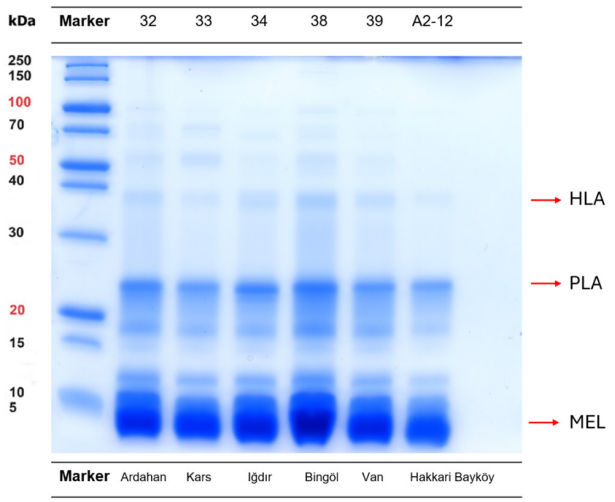

Fig. 10. SDS-PAGE gel imaging of proteins in bee venom for Group 7. Reference bands at 20, 50, and 100 kDa are indicated in red.

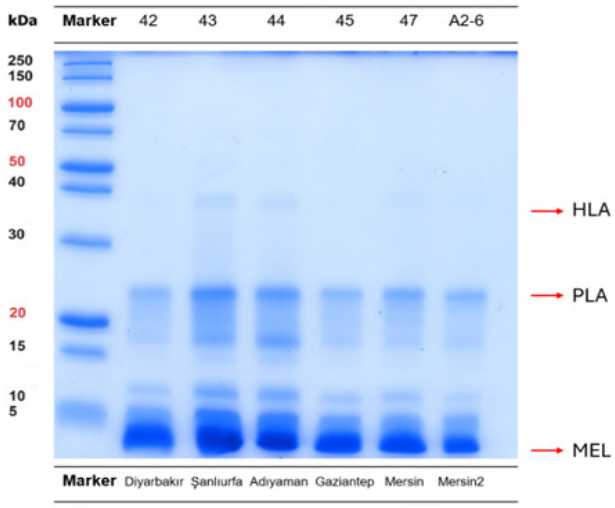

Fig. 11. SDS-PAGE gel imaging of proteins in bee venom for Group 8. Reference bands at 20, 50, and 100 kDa are indicated in red.

DISCUSSION

Apitherapy is a form of alternative therapy that is characterized by the use of such honeybee products as honey, pollen, propolis, royal jelly, and mainly bee venom (BV), also known as apitoxin (Wehbe et al., 2019). Apitoxin is a complex mixture of biologically active substances including proteins, enzymes, and amines (Elieh Ali Komi et al., 2018). Laboratory testing and clinical trials have demonstrated the effectiveness of BV as a biotherapy. In cases of rheumatism and arthritis, BV either prevents inflammation and the breakdown of connective tissues or restores activity and mobility by assisting the body's own defense mechanisms (as in the case of multiple sclerosis and lupus). Recently, the venom has also been investigated as a potential cancer treatment. In addition to its medicinal effects, the researchers suggested that BV may lessen the side effects of various prescription pharmaceuticals (Khalil et al., 2021).

Cancer is a major global public health issue and the second highest cause of death in the United States, despite modern, improved medicines. The number of cancer-related fatalities in the US is predicted to reach 609,360 in 2022, or about 1700 per day. Malignancies of the lung, prostate, and colorectum in males and cancers of the lung, breast, and colorectum in women are responsible for the majority of fatalities (Siegel et al., 2022). Natural products have been thoroughly investigated in the field of drug discovery and have achieved impressive results since they are a rich supply of molecules with a wide variety of structural properties. This is especially the case for the treatment of cancer, where more than half of the authorized medications, such as vincristine, irinotecan, etoposide, and paclitaxel, developed in the last two decades of the 20th century, were derived from natural sources (Huang et al., 2021).

Accordingly, the MTT assay was used in this study to evaluate cancer cell growth after treatment with *Apis mellifera* L. venoms collected from forty-three provinces and fifty-one apiaries and almost all over Türkiye. The crude venom of *Apis mellifera* L. exhibits concentration-dependent cytotoxic effects on selected cell lines, as proven by previous studies (Jang et al., 2003; Ip et al., 2008; Badria et al., 2017; Ceremuga et al., 2020; Duffy et al., 2020; Yaacoub et al., 2021; Borojeni et al., 2021; Kwon et al., 2022). The IC_{50} values, which are used to evaluate the cytotoxicity, were calculated between 1.64±0.70 µg/ml and 98.33±9.28 µg/ml. The samples indicated a statistically significant cytotoxic effect on PC-3, PANC-1, and HeLa cells compared to other cell lines. In addition, A549 and MDA-MB-231 cell lines and U-87 MG, Caco-2, and CCD-24Lu showed statistically similar results. Considering the bee species and ecotypes, protein content

determination, and IC_{50} values, the venoms collected from Maçahel, Faraşin, Kırklareli, Düzce, Adana, Hatay and Gökçeada were found to be the most potent venoms among all samples. Against the venoms, the most sensitive cell lines were PC3, PANC-1 and HeLa cell lines, while the most resistant cell lines Caco-2 and CCD-34Lu exhibited significant cytotoxic effects on the analyzed cancer cells. Our results are similar to those by Babayeva et al. (2024) who reported that some bee venoms collected from a narrower area in Türkiye exhibited significant cytotoxic effects on cancer cells and reported that CARM-L12 TG3, PC-3 and A-673 cell lines exhibited the most significant responses to bee venom. Our findings are consistent with an article by Sivri et al. (2024) comparing the effects of bee venom on MDA-MB-231 and normal breast cells. The study additionally examined at two prometastatic genes, EGFR and WNT7B, and demonstrated that melittin or bee venom can be used in conjunction with an EGFR inhibitor to increase selective anticancer activity. In addition, the IC_{50} values of healthy CCD-34Lu cells showed that all tested venoms were less cytotoxic compared to the positive control agent, doxorubicin, which is an anthracycline medication frequently used to treat a number of malignancies, including Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, sarcoma, thyroid, breast, lung, gastric, ovarian, and pediatric cancers (Thorn et al., 2011). The most important limitation of doxorubicin is that it also affects healthy cells during its use. The findings indicate that the venoms of Turkish bees have a high potential for use as anti-cancer agents. The mechanism of action and selectivity of the venoms will be explained better by further investigations.

Bee venom can activate many anticancer mechanisms simultaneously, especially through such components as melittin and PLA2. The mechanisms of action of melittin, the main component of bee venom, include regulating the cell cycle, altering the permeability of cell membranes, inhibiting proliferation and migration, and promoting endogenous/exogenous apoptosis, autophagy and other regulatory modes of cell death (Mirzaei et al., 2021). It has been reported in the literature that melittin can directly cause necrosis by disrupting cell membrane integrity and activate necroptotic pathways. However, autocaphagic responses are known to be triggered by increased intracellular stress and stimulation of lysosomal pathways and this contributes to cell death. In addition, components such as melittin and phospholipase A2 have been shown to stop the cell cycle in G0/G1 or G2/M phase, thus limiting tumour cell proliferation. Furthermore, it is observed that some components of bee venom cause oxidative stress by increasing the production of reactive oxygen species (ROS) and this contributes to cell death by causing DNA damage and mitochondrial dysfunction (Oršolić, 2012; Stela et al., 2024). Previous research has revealed that bee venom and melittin, its primary active component, exhibit selective cytotoxic effects against various tumor cell lines, particularly those of the prostate, glioblastoma, lung, and breast cancers. These effects were reported to be related to such biomolecules as phospholipase A2 (PLA2) and apamin, present in bee venom, as well as melittin. These antitumour effects of bee venom have been associated with modulation of genes and proteins (e.g., caspases, p53, NF-κB, VEGF, MMPs) that regulate such numerous cellular mechanisms as induction of apoptosis, suppression of proliferation, angiogenesis, and inhibition of metastasis (Oršolić, 2012; Rady et al., 2017). However, despite the therapeutic potential of bee venom, a significant limitation is that it can exhibit undesirable effect as neurotoxicity, haemolysis,, and inflammation, at high doses. In addition, some studies have reported that bee venom has a cytotoxic effect on normal (healthy) cells. Therefore, it is necessary to develop targeted systems and modified formulations to reduce the toxicity of bee venom to healthy cells while increasing its selective activity against cancer cells (El-Didamony et al., 2022; Małek et al., 2023). The cell's innate mechanism for planned cell death is called apoptosis, especially important since it is essential for both development and homeostasis (Pfeffer & Singh, 2018). It is well

known that the ideal method for an anticancer agent to function is to induce the target cancer cells to undergo apoptosis (Ip et al., 2008). According to certain studies, melittin and bee venom both raised the expression and levels of proapoptotic and apoptotic mediators (Khalil et al., 2021).

The characteristics of apoptosis include cellular morphological changes, membrane blebbing, chromatin condensation, oligonucleosomal DNA cleavage, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, dysfunction of the mitochondria, and activation of a family of caspases (Ip et al. 2008). Considering this, annexin V-FITC analysis was performed to identify the apoptotic stage, and the results showed that selected venom samples from Izmir Sasalı, Artvin Macahel and Sırnak Farasin exhibited a concentration-dependent apoptosis-inducing effect on A549 and PANC-1 cells. Apoptosis is a mechanism that several anti-cancer drugs aim to induce; therefore, substances with the ability to stimulate apoptosis have a great potential to be used as anti-cancer agents. Further analysis of the venom's components will help understand the main molecule that causes the apoptosis effect and pave the way for the discovery of new cancer drugs. The study's findings proved that *A. mellifera* venom from Türkiye exhibits potent cytotoxic activity against a variety of human cancer cells. Considering the previous studies, the most abundant component of bee venom, melittin, can be the attributed cause of the cytotoxicity of bee venom (Raghuraman & Chattopadhyay, 2007; Gajski & Garaj-Vrhovac, 2013; Rady et al., 2017; Ağan & Kekeçoğlu 2020; Ceremuga et al., 2020; Duffy et al., 2020). Further studies should be performed on the protein structure, the activity mechanism, and the specificity of the venoms. New findings may clarify the path for the creation of new medications against cancer.

The results of SDS-PAGE analysis of bee venom samples are quite important, especially since the studies in this field are limited. Although results are consistent with previous studies with samples taken from Türkiye (Demir et al., 2011; Şirin et al., 2017); it was possible to visualize a higher number of proteins compared to the mentioned studies. However, to ensure a complete characterization of bee venom samples, it is necessary to use advanced separation techniques such as HPLC and LC-MS.

The SDS-PAGE analysis revealed a higher number of protein bands than previously reported in studies conducted in Türkiye (Demir et al., 2011; Şirin et al., 2017), suggesting a richer and potentially more diverse protein composition in the venom samples. This difference may be attributed to genetic variation among honeybee ecotypes, differences in local floral sources, or variations in venom collection and processing techniques. The presence of additional bands may represent low-abundance peptides or minor enzymatic proteins that were not detected in earlier studies due to limitations in gel resolution or methodology. These could include such molecules as adolapin, secapin, or other bioactive peptides, which contribute to the anti-inflammatory and cytotoxic potential of bee venom (Carpena et al., 2020). The variability in SDS-PAGE profiles observed among samples supports the hypothesis that regional differences in bee populations may result in functionally distinct venom compositions. To build on these findings, future studies should include high-resolution proteomic techniques such as LC-MS/MS to precisely identify and quantify the individual protein components and better understand their biological significance. Based on the results of this study, it is demonstrated that the venom of *Apis mellifera* L.*,* which is collected from fifty-one different locations in Türkiye exhibits dose-dependent cytotoxic and apoptosis-inducing effects. According to the results of protein content and cytotoxic activity, the most effective samples, İzmir Sasalı, Artvin Macahel and Şırnak Farasin BV's were found to have a early apoptosis-inducing effect in a dose-dependent manner. Considering Türkiye'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. Melittin, one of the most abundant components of bee venom, is associated with anticancer

effects in the literature. Further studies are needed to correlate the results obtained in this study with melittin content. In addition, it is known that the melittin peptide is also toxic to normal cells, developing proper delivery vehicles and pharmaceutical formulations or using combination therapies, would be the optimal direction in order to achieve bee venom's therapeutic potential.

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Conflict of interest

The authors declared that there is no conflict of interest.

Ethical issues

The research on honeybees has no ethical issues.

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