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Original Article

In vitro investigation of the cytotoxic effects of various honeybee venoms on Caco-2 and T98G cells

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Abstract

Background: Honeybee (*Apis mellifera*) venom contains several biologically active peptides, including melittin, apamin, enzymes, and non-peptide components. Due to its biologically active components, honeybee venom demonstrates antibacterial, antiviral, anti-inflammatory, and cytotoxic properties. **Aims:** This study aimed to investigate the cytotoxic effects of honeybee venoms collected from different provinces in Turkey on colorectal adenocarcinoma (Caco-2) and glioblastoma multiforme (T98G) cell lines. **Methods:** The apamin, phospholipase A2, and melittin contents of honeybee venoms were analyzed using a high-pressure liquid chromatography (HPLC) variable wavelength detector. The viability of cells after 24-hour exposure to honeybee venoms was determined using the 3-(4,5-dimethylthiazol-2-yl) (MTT), neutral red (NR), and dehydrogenase leakage (LDH) assays. **Results:** Content analysis showed that Mugla had the highest apamin content, while Denizli had the highest phospholipase A2 and melittin contents among the analyzed bee venoms. For Caco-2 cells, the lowest inhibitory concentration (IC_{50}) values were observed in the Denizli venom group, with MTT and LDH results of 12.42 ± 0.19 µg/ml and 8.19 ± 0.61 µg/ml, respectively. For T98G cells, these values were 5.98 ± 0.40 µg/ml and 5.04 ± 0.17 µg/ml, respectively. **Conclusion:** These findings indicate that honeybee venoms from different provinces contain varying levels of apamin, phospholipase A2, and melittin. The cytotoxic effects observed on Caco-2 and T98G cell lines suggest that honeybee venom may have potential as an anticancer agent.

Key words: *Apis mellifera*, Cell culture, Cytotoxicity, HPLC, MTT

Introduction

Honeybees adapt to regional ecological conditions, and it is known that there are various ecotypes and races in Anatolia (Kence, 2006). *Apis mellifera anatoliaca* is a native honeybee, except in the southeast and northeast regions of Turkey (Sıralı, 2017). *Apis mellifera caucasia*, distributed across the Artvin and Ardahan provinces, has been determined as the gene center of the Caucasian bee breed in Turkey (Aygun and Akbulak, 2017). It has been reported that the honeybees in southern Thrace are generally *A. mellifera anatoliaca*, whereas honeybees closely related to *Apis mellifera carnica* are generally in northern Thrace, with some mixing (Cakmak *et al*., 2014). *A. mellifera syriaca* is found around Hatay and the Syrian border of Turkey, whereas *Apis mellifera meda* is found around Lake Van in Eastern Anatolia and reaches to Elazig, Tunceli, and Malatya (Ruttner *et al*., 1985; Smith *et al*., 1997). Apitherapy, a traditional healing practice that utilizes honeybee products for disease treatment and prevention, has recently regained popularity (Senel and Demir, 2018; Ozdemir *et al*., 2021). Bee venom, just one of many bee products, consists of alarm pheromones secreted by honeybees as a defense mechanism against possible attacks by other bees (Bovi *et al*., 2017; Caprazlı and Kekecoglu, 2021). It contains water, phospholipase A2, phospholipase B, hyaluronidase, α-glucosidase, acid phosphomonoesterase enzymes, melittin, apamin, MCD, secapin, tertiapine, adolapin peptides, active amines, amino acids, sugars, lipids, minerals, and volatile compounds (Altıntas and Bektas, 2019). Studies have reported that bee venoms have radioprotective (Gajski and Garaj-Vrhovac, 2009), anti-inflammatory (Sobral *et al*., 2016), antinociceptive (Baek *et al*., 2006), and anticancer properties (Park *et al*., 2001) due to these pharmacologically valuable components.

Colorectal cancer is one of the types of cancer most associated with high mortality and morbidity (Haggar and Boushey, 2009). Likewise, glioblastoma is a common and aggressive malignant brain tumor (Fabiani, 2020), and it has limited treatment options due to its complex structure. It has been reported that bee venom and its main component, melittin, show anticancer effects on cancer cells by causing apoptosis or suppressing metastasis and proliferation (Oršolić, 2012).

The study aimed to evaluate the potential cytotoxic effects of honeybee venoms on colorectal cancer cell lines (Caco-2) and glioblastoma multiforme cell lines (T98G).

Materials and Methods

Collection of honeybee venom samples

Apis mellifera bee venom samples (10 mg) were collected from Afyon, Ankara, Balıkesir, Denizli, and Mugla in July and August 2021 using a bee venom collector (Beesas Beekeeping, Turkey). The collector transmits a mild electrical shock to the bees, their venom falls onto glass plates below, and it is scraped off using a lancet. The bee venom was subsequently freeze-dried and stored in a freezer at -70°C until analysis (Sevin *et al*., 2022).

Content analysis of honeybee venom by high-pressure liquid chromatography

A content analysis of all venom samples was carried out at the Mugla Sıtkı Kocman University Food Analysis Application and Research Center. The samples were analyzed for apamin, phospholipase A2, and melittin by using an Agilent 1260 HPLC variable wavelength detector (VWD). The column flow rate was 1 ml/min, and the optimum temperature was 20°C. Standard solutions of apamin, phospholipase A2, and melittin were prepared at concentrations of 10, 20, 50, and 100 µg/ml. 0.1% TFA in H_2O and 0.1% TFA in acetonitrile were used as mobile phases. The method was adapted from a study by Frangieh *et al.* (2019).

Cell culture and treatment

The cell culture experiments of the study were performed in the *in vitro* Toxicology Laboratory of the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University.

The Caco-2 (ATCC® HTB-37™) and T98G (ATCC® CRL-1690™) cell lines were cultured using Dulbecco's Modified Eagle Medium (DMEM) (BioInd, Israel) with 10% fetal bovine serum and 1% penicillin-streptomycin (10.000 U/ml penicillin and 10 mg/ml streptomycin) (BioInd, Israel) in 75-cm² tissue culture flasks (Sarstedt, Germany) and grown at 37°C in a humidified carbon dioxide incubator at 5% CO_2 (Nuve EC160, Turkey). The honeybee venoms were dissolved in dimethyl sulfoxide (DMSO), and the obtained stock solutions and half concentrations were prepared using the DMEM cell culture medium. Cells on the flask surface were removed with 0.25% v/v trypsin/EDTA (Capricorn, Germany), and the cells were seeded at an approximate density of 2×10^3 cells/well in 96-well plates. The cells were left to adhere in an incubator for 24 h and then expanded to approximately 80% confluence in 96-well plates. The cell density was automatically calculated using a Julli FL LiveCell Analyzer (NanoEnTek Inc., Seoul, South Korea). The cells were incubated as a negative control (only medium), a positive control (Triton X), a solvent control (DMSO), and the experimental groups (bee venom). Treatments at each dose were conducted as three replicates on the same plate.

Cell viability assays

After 24 h of incubation, 15 µL 3-(4,5-dimethylthiazol-2-yl) (MTT) solution was added to each well and the microplates were incubated at 37°C for 4 h. After the formazan was dissolved using 100 µL DMSO, absorbance was measured at 540 nm with a microplate reader (Spectra Max i3/i3x Multi Mode Detection Platform, Molecular Devices, Sunnyvale, CA, USA) (Mosmann, 1983; Arslan *et al*., 2021).

Following 24 h of incubation, 100 µg/ml NR solution (40 µg/ml DMEM) was added to each well and incubated at 37°C for 2 h. The wells were then washed with phosphate-buffered saline and combined with 100 µL of lysis solution (1% acetic acid, 50% ethanol, 49% H_2O) in a shaker for 20 min. The absorbance of the converted dye was measured at 540 nm using the same microplate reader as for the MTT assay.

A commercial test kit (ChronoLab Quantitative Detection of Lactate Dehydrogenase, Barcelona, Spain) was used to determine LDH activity. Following 24 h of incubation, the culture media were separated, and the test reagent was added to this media. The optical density was determined at 340 nm. To determine the cytotoxicity, the negative control group, which was set to 100% viability, and the positive control group, which was treated with Triton-X and set to completely dead, were compared.

Statistical analysis

The cytotoxicity percentage for each venom sample and its concentration was calculated using Microsoft Excel. The IC_{50} values were calculated using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance (SPSS Inc., Chicago, IL, USA) was done for the MTT, NR, and LDH assays in terms of dose, the province where the venom was obtained, and the cell line. Descriptive statistics for categorical data were analyzed using frequency and percentage, while mean and standard deviation were used for continuous data. The distribution of the data was determined using the Kolmogorov-Smirnov/Shapiro-Wilk test statistics. Independent sample t-test and ANOVA were used to compare differences in the data. Correlation analysis was used to determine the relationship between variables. The correlation analysis were interpreted as absolute values; between 0.70-1.00 as a high-level relationship, between 0.70-0.30 as a medium-level relationship, and between 0.30-0.00 as a low-level relationship (Büyüköztürk, 2009).

Results

According to HPLC analysis, the lowest apamin content was found in Afyon and the highest was in Mugla. The lowest proportion of phospholipase A2 was found in Mugla and the highest was in Denizli, followed by Balıkesir, Afyon, and Ankara. The highest melittin content was from Denizli and the lowest was from Afyon

Table 1: The amounts of apamin, phospholipase A2 and melittin (%)

Substance	Sample				
	Afyon	Ankara	Balıkesir	Denizli	Muğla
Apamin (%)	3.84	5.00	3.89	4.42	6.29
Phospholipase A2 (%)	11.76	11.17	12.96	13.87	10.87
Melittin (%)	51.17	63.92	65.50	70.21	57.13

Table 2: IC_{50} values (µg/ml) of bee venom samples by MTT, NR, and LDH assay (mean±SD)

Sample	MTT		NR		LDH	
	Caco-2	T98G	Caco-2	T98G	Caco-2	T98G
Afyon	18.08±1.31	8.53±0.42	14.72±0.35	10.35±0.97[a]	19.52±0.58	9.29±0.50
Ankara	16.75±0.53	7.70±0.32	13.59±1.29	8.57±0.59[ab]	13.67±0.85	10.64±0.34
Balıkesir	15.13±1.36	6.49±0.34	11.47±0.42	5.74±0.42[ab]	11.59±0.38	6.94±0.40
Denizli	12.42±0.19	5.98±0.40	13.25±0.77	4.90±0.21[b]	8.19±0.61	5.04±0.17
Muğla	16.56±0.95	7.29±0.58	14.51±0.84	8.32±0.49[ab]	14.43±0.68	9.18±0.54
P-value	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05

[a, b] Indicates the province where no difference was observed

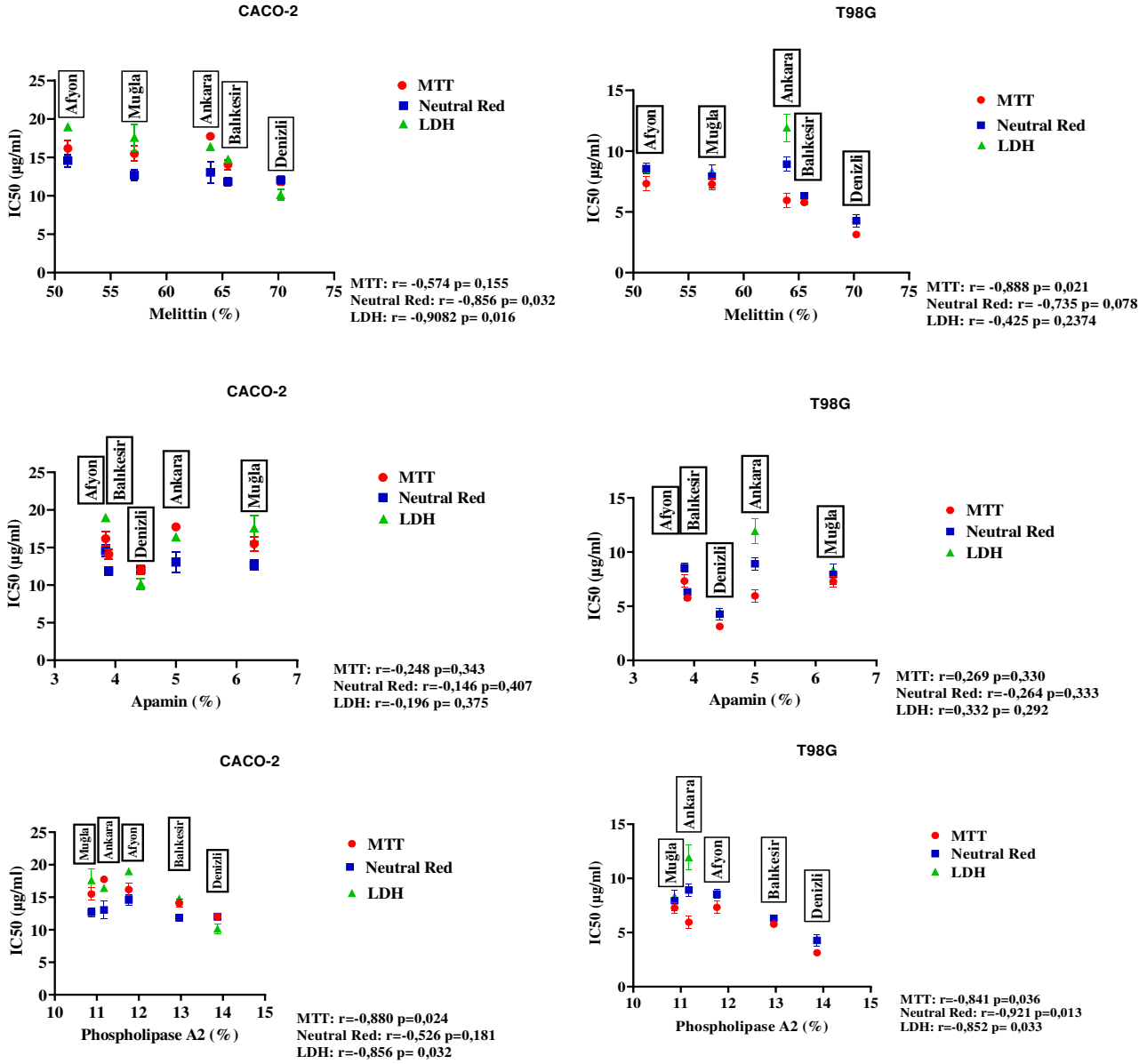
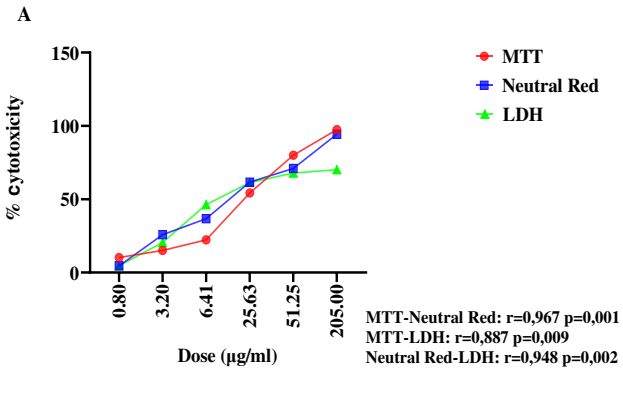
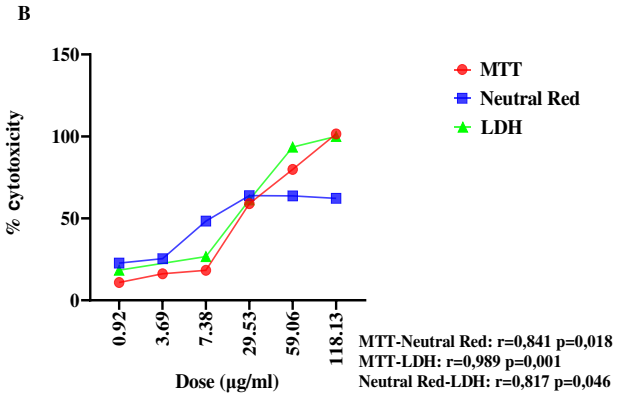
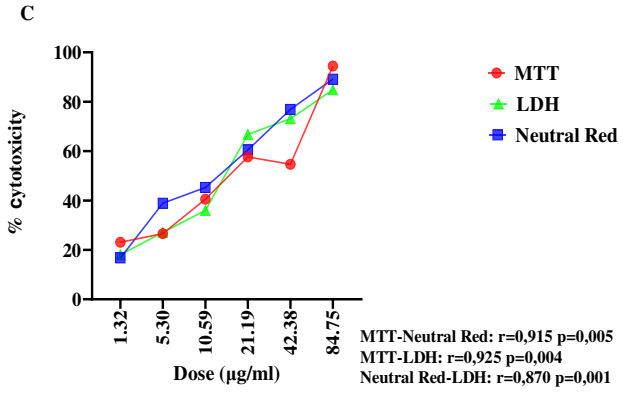
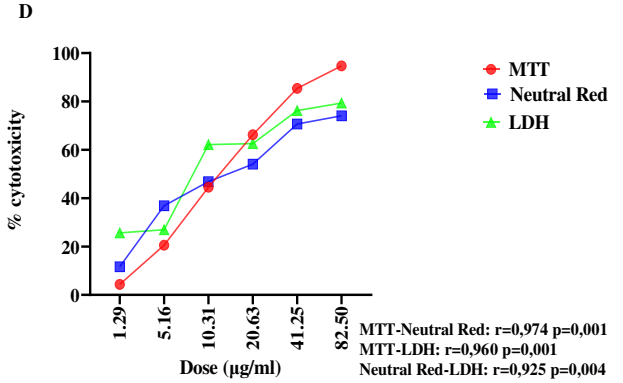


Fig. 1: The relationship between the different amounts of apamin, melittin, and phospholipase A2 in bee venoms and the IC_{50} values of the cells









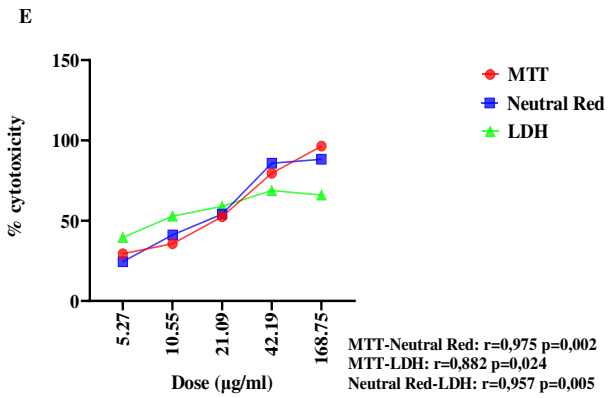
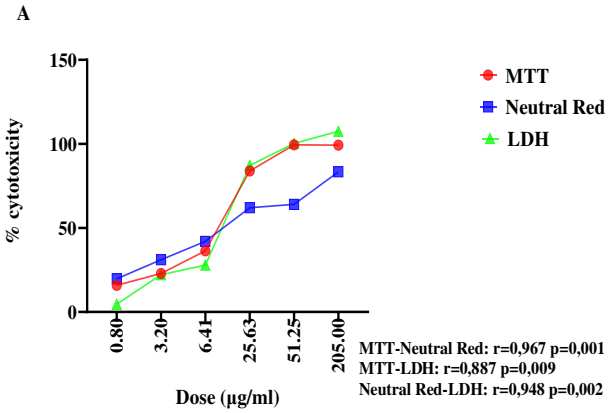
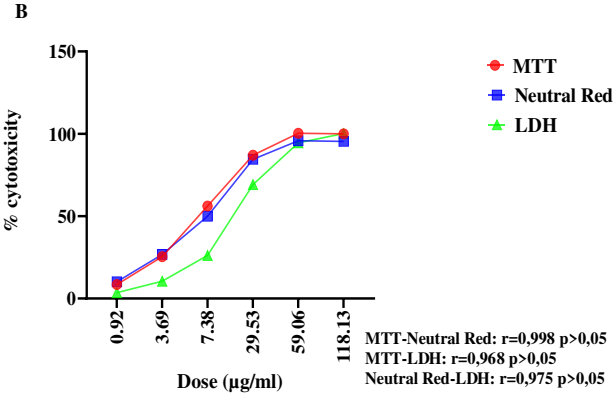
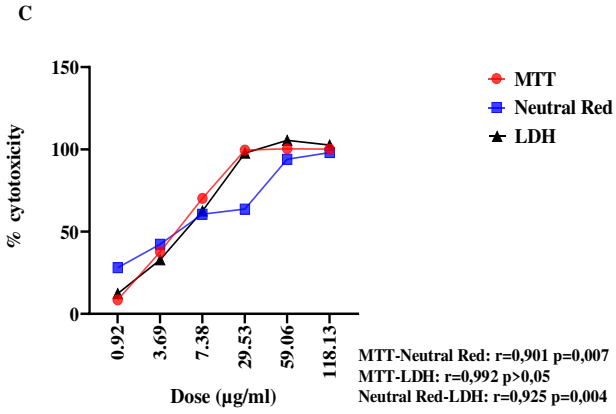


Fig. 2: Relationship between % cytotoxicity values determined according to dose by MTT, Neutral Red, and LDH analyses in Caco-2 cells (r Correlation Coefficient, P<0.05 Significance of differences between doses according to methods. **A**: Afyon, **B**: Ankara, **C**: Balıkesir, **D**: Denizli, and **E**: Muğla)







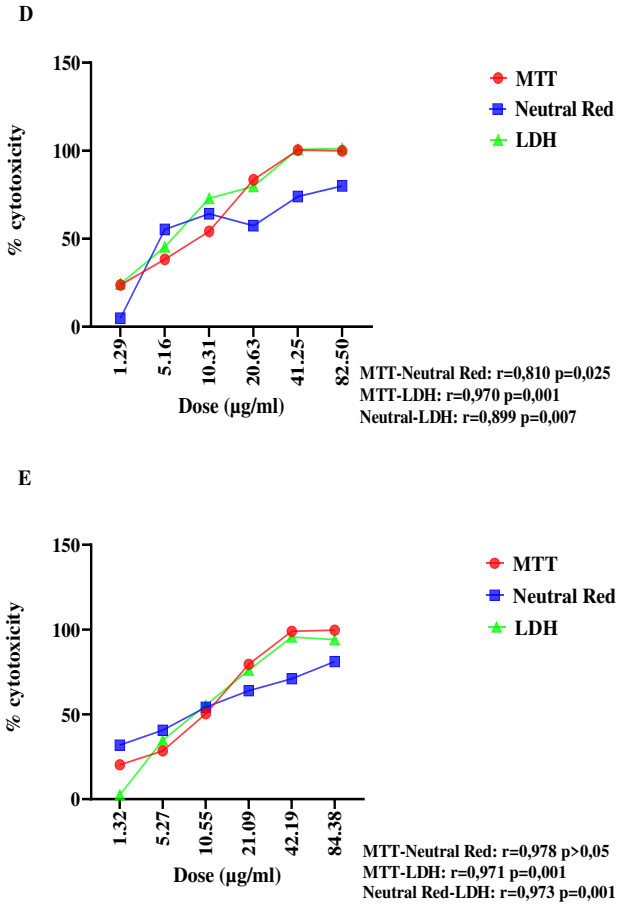


Fig. 3: Relationship between % cytotoxicity values determined according to dose by MTT, Neutral Red, and LDH analyses in T98G cells (r Correlation Coefficient, P<0.05 Significance of differences between doses according to methods. **A**: Afyon, **B**: Ankara, **C**: Balıkesir, **D**: Denizli, and **E**: Muğla)

(Table 1).

According to the MTT assays, the highest IC_{50} value for both cell lines was from the Afyon venom and the lowest was from Denizli. The distribution of bee venom from Afyon and Denizli showed homogenity, as indicated by similar IC_{50} values calculated by MTT and LDH assays in all repeated analyses. However, statistically significant differences were observed in IC_{50} values for T98G cells in the NR assay when comparing provinces (P<0.05). The analysis identified Afyon and Denizli as the provinces contributing to this difference. No significant differences were detected in the other assays (P>0.05) (Table 2).

In NR, the highest IC_{50} value for both cell lines was from the Afyon venom, whereas the lowest for Caco-2 was in Balıkesir, and the lowest for T98G was in Denizli. At least in the LDH assay, the highest IC_{50} value for Caco-2 and T98G cells was found in the honeybee venom of Afyon and Ankara, respectively, whereas the lowest for both cell lines was in Denizli. The IC_{50} values of all the honeybee venoms are shown in Table 2.

For the Caco-2 cell line, a negative correlation was observed between the different melittin amounts of all bee venoms and the IC_{50} values calculated from all analysis methods, and the negative correlation was statistically significant for the IC_{50} values from the NR and LDH analyses (P<0.05). A positive correlation was found between the different apamin amounts of all bee venoms and the IC_{50} values calculated by MTT and LDH, whereas a negative correlation was found for the NR assays. A negative correlation was found between all phospholipase A2 samples and the IC_{50} values calculated from all analysis methods, with a statistically significant negative correlation for the IC_{50} values from the MTT and LDH analyses (P<0.05) (Fig. 1). For the T98G cell line, a negative correlation was observed between the different melittin amounts of all bee venoms and the IC_{50} values calculated from all analysis methods, and the negative correlation was statistically significant for the IC_{50} values from the MTT assay (P<0.05). A positive correlation was found between the different apamin amounts of all honeybee venoms and the IC_{50} values calculated from all analysis methods. A statistically significant negative correlation was found between all phospholipase A2 samples and the IC_{50} values calculated from all analysis methods (P<0.05) (Fig. 1).

There was 57%, 86%, and 91% negative correlation between the IC_{50} values calculated using MTT, NR, and LDH assays and the melittin content of bee venoms for Caco-2 cells, respectively. For T98G cells, there was an 88%, 73%, and 42% negative correlation, respectively. In Caco-2 cells, a moderate level of correlation was calculated for MTT, while a high level of correlation was found for NR and LDH assays. In T98G cells, a high level of correlation was observed for MTT and NR assays, while a moderate level of correlation was found for the LDH assay. For Caco-2, between the apamin content of bee venoms calculated by MTT, NR, and LDH assays and IC_{50} values, a negative correlation of 25% was observed with MTT, 15% with NR, and a negative and low correlation of 20% with LDH. For T98G, a positive correlation of 27% was found with MTT, 33% with LDH, and a negative correlation of 26% with NR. In T98G cells, a low level of correlation was calculated for MTT and NR assays, while a moderate level of correlation was found for the LDH assay.

For Caco-2 cells, a negative correlation of 88%, 53%, and 86% was found between the phospholipase A2 content of bee venoms and the IC_{50} values calculated using MTT, NR, and LDH assays, respectively. For T98G cells, a negative correlation of 84%, 92%, and 85% was observed, respectively. In Caco-2 cells, a high level of correlation was calculated for MTT, while a moderate level was found for NR and LDH assays. In T98G cells, a level of correlation was observed for all assays.

For Caco-2 and T98G cells, cytotoxicity changes were correlated with the dose of bee venom samples (Figs. 2 and 3). Graphs were drawn separately according to MTT, LDH, and NR assays, and when evaluated in general, similar relationships were found between cytotoxicity values. The highest correlation was between MTT and LDH and MTT and NR for both Caco-2 and T98G cells in the bee venom from Ankara, respectively,

with a positive correlation of approximately 99%.

Discussion

Natural products, which are very rich in chemical components, have been investigated for many years for their protective properties against cancer (Huang *et al*., 2021). In recent years, extensive studies have been conducted on the use of natural products such as biotoxins in cancer treatment (Lee *et al*., 2015; Zheng *et al*., 2015). Several studies reported that bee venom and its components induce cell cycle arrest, apoptosis, and growth inhibition of different types of cancer cells (Oršolić *et al*., 2003; Moon *et al*., 2006; Ip *et al*., 2008). In this study, the cytotoxic effect of honeybee venoms obtained using an electroshock device was investigated. The bee venom samples were obtained from regions with very active beekeeping.

Bee venom can be obtained using different methods, including dissection of the venom sacs, retrieving venom after inducing the bees to sting, and using venom collection devices (Caprazlı and Kekecoglu, 2021). One study used different collection methods, including electroshock and dissection of the venom sacs, to obtain venom from Carniolan and Italian bees. The dissection method showed higher inhibition on liver (Hep-G2), breast (MCF-7), and colorectal cancer cell lines than the electroshock method due to retaining a higher amount of protein (Badria *et al*., 2017). However, the electroshock method is often preferred over the dissection method because it is more practical, and the venom is taken at the same time from all the bees.

As is common in natural products, the components of bee venom can vary depending on many factors, and their percentages can be determined using HPLC (Rybak-Chmielewska and Szczesna, 2004; El-Wahed *et al*., 2019).

In a content analysis study comparing freshly obtained Anatolian honeybee venom and commercially purchased honeybee venom using HPLC-UV, it was stated that the fresh bee venom samples were richer in apamin, melittin, and phospholipase A2 than the commercial bee venom (Samancı and Kekecoglu, 2019). In another study that collected bee venom samples from different regions of Anatolia and analyzed them using HPLC-UV, the melittin amount in the samples was in the range of 26.76%-51.85%, the phospholipase A2 was 9.26%-17.83%, and the apamin was 1.40%-2.85% (Tanugur and Kekecoglu, 2021). The results of this study showed that the bee venom from different regions can have different amounts of melittin, apamin, and phospholipase A2, possibly because of the race of the bee, the climate of the region where the bee lives, the vegetation, and the geographical conditions.

In a study that investigated the anticancer properties of bee venom and its components on Hs683, T98G, and U373 human glioma cells, an MTT-based cell viability assay revealed bee venom IC_{50} values of 7.12, 15.35, and 7.60 µg/ml, respectively (Lebel *et al*., 2021). In another study, the IC_{50} value after 24 h of bee venom treatment by MTT assay was determined to be 28.53 µg/ml (Bazi *et al*., 2015). Our results showed variable IC_{50} values determined from all venom samples using the same viability assay due to factors such as geographical region, climate, race/subspecies, chemical properties of the solvents used in the extraction and dissolution of the bee venom, and/or different laboratory conditions. It was found that Denizli has bee venom with the highest melittin and phospholipase A2 content. Its high cytotoxic activity may be related to these substances, but other bee venom components, especially apamin, may also have cytotoxic effects. According to the results of all three analytical methods, it was observed that the venom might cause cytotoxicity by affecting mitochondrial signaling pathways, cell membrane structure, and/or cell metabolism, and it may also cause lysosomal damage. Considering the IC_{50} values, it can be understood that the cytotoxic activity on Caco-2 cells is lower than that on T98G cells. It is thought that T98G cells are more sensitive to honeybee venom due to the specific metabolic activity and physical structure of the cells.

Recent research has demonstrated that bee venom may possess synergistic activity in combination with chemotherapeutic drugs, a phenomenon that appears to be especially evident in drug-resistant tumors. Consistent with this, this compound has exhibited potent antitumor activity against cervical carcinoma and laryngeal carcinoma cells and their drug-resistant variants, indicating that bee venom may find application in circumventing the drug resistance mechanisms that arise as a result of chemotherapy (Gajski *et al*., 2014). Furthermore, melittin has been found to enhance the sensitivity of paclitaxel-resistant ovarian cancer cells to drug activity by regulating apoptosis-related signaling pathways (Son *et al*., 2007). Collectively, these results suggest that bee venom will increase sensitivity to chemotherapy drugs and is an effective adjuvant to include in combination therapies.

The prospective therapeutic potential of bee venom has been a subject of great interest, especially because of its cytotoxicity against cancer cells, which has instigated the initiation of numerous preclinical studies in this area. Nevertheless, the prospective toxic actions of bee venom and its components on non-tumor cells are regarded as the biggest drawback to its therapeutic application. In this regard, some *in vitro* studies investigated the genotoxicity of bee venom on normal cells. Specifically, it was reported that human peripheral blood lymphocytes treated with high concentrations of bee venom experience considerable cytogenetic damage characterized by chromosomal aberrations, micronucleus occurrence, and reduction of mitotic index (Garaj-Vrhovac and Gajski, 2009). Likewise, bee venom has also been documented to cause DNA damage, decrease cell viability, and trigger apoptosis in human leukocytes (Gajski and Garaj-Vrhovac, 2011). Furthermore, research demonstrates that melittin is capable of triggering oxidative stress and altering gene expression patterns of human peripheral blood lymphocytes (Gajski *et al*., 2016). It has been found that melittin represses the

transcription of DNA repair pathway genes and induces cell cycle arrest (Lee and Bae, 2016). The results point to the potential of bee venom and its constituents in modulating both cancer and non-cancer cells and demonstrate the necessity of a careful and comprehensive evaluation process in potential therapeutic use (Sjakste and Gajski, 2023).

In summary, the potential cytotoxic effect of bee venoms on cancer cells was evaluated using *in vitro* cell culture studies, and it was observed that honeybee venom inhibited the proliferation of Caco-2 and T98G cells in a dose-dependent manner. A positive correlation was found between increased doses of honeybee venom and cytotoxicity (%) in the cell lines, parallelism was seen in the responses of the doses in different viability assays, and correlations were found between the methods. Due to its components, honeybee venom can affect cancerous cells. Considering its cytotoxic effect, it has the feature of being a potential anticancer agent for cancer treatment. However, the use of bee venom is limited due to its direct toxic effect, and extensive preclinical studies are required to develop its therapeutic use.

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Conflict of interest

The authors declared that there is no conflict of interest.

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