Title

Assessment of Iranian Bee venom from diverse climate zones and seasons for effects on cancer cell lines

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Abstract

Introduction

Honey bee (*Apis mellifera*) venom (apitoxin) is a promising natural anticancer agent due to bioactive components like melittin, phospholipase A_2 (PLA_2), and hyaluronidase. Its composition and activity are shaped by environmental factors such as climate, temperature, humidity, and season.

Objective

This study characterized the biochemical properties and in vitro anticancer activity of bee venom collected in spring and autumn 2023 from five climatically diverse Iranian provinces: Alborz (cold semi-arid), Ardabil (cold mountainous), Fars (hot semi-arid/warm wet), Hormozgan (hot desert/arid), and Mazandaran (humid subtropical/coastal).

Materials and Methods

Venom was collected non-lethally via electric stimulation. Composition was analyzed using SDS-PAGE, RP-HPLC (melittin quantification), and mass spectrometry. Biological activity was assessed via hemolytic, colorimetric PLA_2, and turbidometric hyaluronidase assays, plus MTT cytotoxicity testing on six human cancer cell lines (AGS, Panc-1, DLCL-2, HT-29, HepG2, A549).

Results

Melittin content ranged from 59.25–72.85% dry weight, peaking in Hormozgan autumn samples (72.85%)—above typical global ranges (40–60%). Fars showed the strongest seasonal effect (~20% autumn increase). PLA_2 and hyaluronidase activities were generally higher in autumn. Cytotoxicity was greater in autumn venoms, especially from warmer regions (Hormozgan, Fars), with CC_{50} values <1–2 µg/well against DLCL-2, AGS, and Panc-1, correlating with melittin levels. Hemolytic activity stayed consistent across samples.

Conclusion

Iranian bee venom shows potent in vitro anticancer activity, varying by region and season, favoring autumn collections from hot-arid zones—supporting region-specific harvesting for oncological applications.

Keywords: Apitoxin, Carcinomas, climate,Enzyme , melittin, Seasonal periods

1.Introduction

Nature provides a rich reservoir of therapeutic compounds, offering solutions for treating diseases and alleviating symptoms. Plants, bacteria, fungi, and insects produce diverse bioactive molecules that have underpinned traditional medicine for centuries. Among these, bee-derived products stand out for their chemical complexity, delivering potent, abundant, and sustainable therapeutic agents. The honey bee, *Apis mellifera*, one of the major pollinators of ecosystems natural and agricultural, produces bee venom, or apitoxin—a rich mixture synthesized in its venom gland and delivered via its sting (1). First considered a defense mechanism, this nonvolatile, colorless liquid, pH 4.5–5.5, contains primarily water (88%) and trace quantities of active ingredients (0.1–0.2 µg per sting) (2). Bee venom has a broad variety of enzymes, peptides, amines, and other bioactive molecules responsible for its therapeutic use (3).

Bee venom of *Apis mellifera* is a complex mixture containing melittin, phospholipase A_2, hyaluronidase, histamine, catecholamines, and serotonin, all contributing to its biological activity (2,6,26). These components act synergistically to produce antimicrobial, inflammatory, and cytotoxic effects (1,3). Among them, melittin represents the major peptide fraction and is primarily responsible for the venom's therapeutic and anticancer properties (4,5).Melittin is a peptide forming 50–60% of the venom dry weight and the primary active constituent. The 2.84-kDa peptide induces membrane disruption, increases smooth muscle contraction, increases capillary permeability, and possesses anticoagulant activities, although its action has the potential to be problematic by inducing side effects (4,5). The higher molecular-weight units, such as phospholipase A2 (19 kDa, 128 amino acids) and hyaluronidase (38 kDa), are allergenic and hence it becomes challenging to apply them clinically (2,6).

The protein content of bee venom is considerably influenced by numerous factors such as age, strain, social position, geographic location, season, and climate (7,8). Temperature, of environmental factors, has an extremely significant effect on the venom's protein profile, hence its biological activity (9). Several studies, including Iranian studies, have revealed a wide array of pharmacological activities such as immunomodulatory, anti-inflammatory, analgesic, antiapoptotic, and antiarthritic activities (10–14). Primarily, anticancer potential of bee venom, through melittin's ability to lyse cancer cell membranes and induce apoptosis, has been of

widespread interest owing to its therapeutic promise (15,16). Melittin's activity, due to its potential for the selective targeting of cancer cells, renders it an eventual candidate for novel cancer treatments (5,17). Venom is most commonly gathered using electric stimulation, with yield and quality varying according to season, age of colony, feeding, and geographic location (8,18).

Iran's diverse geography—from deserts, mountainous areas, and wetland coastal regions—has made it a honeybee hub throughout history. The identification of Iranian bee venom to tap into its curative potency, particularly in the field of oncology, has been the target of new research (19,20). Variation of venom composition across Iran's climatic regions highlights region-specific studies to establish its biochemical and therapeutic attributes (21). Seasonal variations in honey bee venom composition have been identified, including the expression of an antigen 5-like protein in winter but not summer venom glands, highlighting the influence of seasonality on venom components and their potential biological activities (22).

This study investigates Iranian bee venom collected from diverse climate zones, characterizing it and exploring its biological activity, focusing particularly on its anticancer properties against multiple cancer cell lines. By elucidating the influence of climate and seasonality on venom composition, this research attempts to bridge gaps in regional differences and therapeutic potential, hoping to offer contributions to new cancer therapy methods.

2.Material and methods

2.1.Venom collection

To explore the therapeutic potential of bee venom, we traveled across Iran’s varied regions Alborz’s semi-arid plains(35.8355°N, 50.9515°E, ≈1,300 m), Ardabil’s cool, mountainous terrain(38.2495°N, 48.2933°E, ≈1,350 m), Fars’ warm, fertile valleys (29.5926°N, 52.5837°E, ≈1,500 m), Hormozgan’s arid deserts(27.1832°N, 56.2666°E, ≈10 m), and Mazandaran’s humid coastal areas(36.5633°N, 53.2793°E, ≈50 m) during spring (March–April) and autumn (September–October) of 2023. In each province, we selected three apiaries, each housing 10–15 healthy *Apis mellifera* hives, to capture venom influenced by Iran’s diverse climates. We employed a specialized electric shock device (Asgharpour, Iran; 12V, 0.5 Hz pulses) placed at the hive entrance for 45 minutes to gently prompt bees to release venom onto a glass plate,

ensuring their safety. After collection, the plate was cooled at 4°C for 10 minutes to preserve venom quality, then carefully scraped with a sterile scalpel into clean glass containers. Each apiary yielded 100–120 mg of dry venom, with 10–12 samples per province per season, stored at -20°C and transported to the laboratory within 24 hours to maintain bioactivity.

We employed a specialised electric stimulation device (Asgharpour, Iran; 12 V, 0.5 Hz pulses) placed at the hive entrance for 45 minutes to gently stimulate bees to deposit venom onto a glass plate without harming them. After collection, the plate was cooled at 4 °C for 10 minutes, and dried venom was carefully scraped with a sterile scalpel into clean glass containers. Each apiary yielded 100–120 mg of dry venom. Samples (10–12 per province per season) were immediately frozen at −20 °C and transported to the laboratory within 24 hours to preserve bioactivity. This method followed established non-lethal protocols (Ferreira Junior et al., 2010).This method, adapted from established protocols (9), ensured consistent, high-quality samples for analyzing venom's composition and cancer-fighting potential.

2.2.SDS-PAGE

To investigate the protein composition of bee venom, we analyzed all samples using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), following the established protocol by Laemmli (23). We prepared crude venom samples (10 µg per lane) in a non-reducing sample buffer, omitting reducing agents such as β-mercaptoethanol to maintain disulfide bonds and preserve the native structure of venom proteins. These samples were separated on 12% polyacrylamide gels using a Mini-PROTEAN system (Bio-Rad). To visualize the proteins, we employed Coomassie Brilliant Blue R-250 for standard detection. Electrophoresis was conducted at 120 V for 90 minutes, with a molecular weight marker (Sinaclone) included to estimate protein sizes. This approach enabled us to identify key venom components, such as melittin, and to compare protein profiles across samples collected from diverse regions and seasons in Iran.

2.3.RP-HPLC and mass analysis

To characterize the composition of bee venom and quantify its primary component, melittin, we employed reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. Crude bee venom samples, collected from five Iranian provinces, were reconstituted in ultrapure water to a concentration of 1 mg/mL and filtered through a 0.2 µm

filter (Sartorius, Germany) to remove particulates. For analytical RP-HPLC, we used an Agilent 1200 HPLC system equipped with a quaternary pump (DE62963133), an automatic injector, and a multi-wavelength detector. We injected 100 µL of each sample onto a C18 reverse-phase column (100 Å, 5 µm, 4.6 × 150 mm), pre-equilibrated with solvent A (water with 0.1% trifluoroacetic acid, TFA). Proteins were eluted using a linear gradient from 0% to 80% solvent B (80% acetonitrile, 0.1% TFA) over 40 minutes at a flow rate of 1 mL/min, with detection at 214 nm to monitor peptide bonds. Chromatograms were generated for all venom samples collected in spring and autumn, and the retention time of melittin was validated using a commercial standard (Sigma, Cat. No. 20449-79-0), which eluted at approximately 30 minutes (see Figure 2).

To isolate larger quantities of melittin for further analysis, we performed semi-preparative HPLC using a Knauer system (Germany) fitted with a C18 column (10 µm, 120 × 20 mm). The column was equilibrated with solvent A (water, 0.1% TFA), and samples were loaded (quantity per run optimized based on sample concentration) before elution with a gradient of 5% to 100% solvent B (80% acetonitrile, 0.1% TFA) over 40 minutes, monitored at 214 nm. The melittin fraction, eluting between 65 and 70 minutes, was collected, lyophilized into a powder, and stored at -20°C. The purity of the isolated melittin was confirmed using analytical RP-HPLC, as described above (see Figure 3).

For molecular characterization, we conducted mass spectrometry using a Waters Alliance 2695 HPLC system coupled to a Micromass Quattro micro API mass spectrometer. Samples were separated on an Atlantis T3-C18 column (3 µm, 2.1 × 150 mm) at a flow rate of 0.2 mL/min with a 5 µL injection volume. The mobile phase consisted of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid) in a 60:40 linear gradient. Mass spectrometry was performed in positive electrospray ionization (ESI+) mode with the following parameters: cone voltage, 30 V; capillary voltage, 4 kV; extractor voltage, 2 V; RF lens, 0.2 V; collision energy, 30 eV; nebulizer gas (N_2) flow, 200 L/h; source temperature, 120°C; and desolvation temperature, 300°C. This analysis confirmed the molecular identity of purified melittin, with mass spectra matching the standard melittin profile (supplementary files).

2.4.Hemolytic assessment

To evaluate the hemolytic potential of bee venom and its primary component, melittin, we assessed their ability to lyse red blood cells (RBCs) using a modified version of the method described by Yousefpoor et al. (2019) (24). Fresh, healthy human blood (5 mL), obtained with ethical approval, was mixed with 10 mL of heparin to prevent coagulation. The blood was centrifuged at 3500 rpm for 10 minutes to separate plasma and lysed cells, leaving a pellet of RBCs. We washed the RBC pellet repeatedly with phosphate-buffered saline (PBS) until the supernatant was clear, discarding the supernatant after each wash. The resulting RBCs were resuspended in PBS to create a 2% (v/v) suspension.

For the assay, we serially diluted venom samples in PBS to achieve concentrations ranging from 0.0625 to 4 µg/mL in a 96-well plate, with each well containing 100 µL of diluted sample. We then added 100 µL of the 2% RBC suspension to each well, resulting in a final volume of 200 µL. Positive controls (100 µL PBS + 100 µL 2% RBCs, representing no lysis) and negative controls (100 µL 1% Triton X-100 + 100 µL 2% RBCs, representing complete lysis) were included in separate rows for simultaneous analysis. The plate was incubated at 37°C for 2 hours, followed by centrifugation at 3500 rpm for 10 minutes to pellet intact RBCs. We transferred 100 µL of each supernatant to a new 96-well plate and measured the absorbance of released hemoglobin at 540 nm using a microplate spectrophotometer (Dynex MRX).

To ensure reliability, all assays were performed in triplicate. The percentage of hemolysis was calculated using the following equation, with PBS as the reference buffer:

%hemolysis = Sample absorption − Negative control absorption / Positive control absorption − Negative control absorption x100

2.5.Hyaloronidae activity

To explore the enzymatic potential of bee venom samples, we conducted a hyaluronidase activity assay with meticulous attention to detail. The process began by carefully preparing several essential reagents. We started with a 300 millimolar sodium phosphate monobasic solution, derived from a 5.0 molar stock, and fine-tuned its pH to 5.35 at 37 degrees Celsius. Next, we crafted a hyaluronic acid solution using hyaluronic acid (Sigma, H5388) at a concentration of 0.3 milligrams per milliliter within the 300 millimolar phosphate buffer. This mixture was gently heated to 90–95 degrees Celsius while stirring until the hyaluronic acid completely dissolved, with the pH held steady at 5.35 at 37 degrees Celsius using either 1 molar

sodium hydroxide or 1 molar hydrochloric acid as required. The enzyme diluent was thoughtfully composed of 20 millimolar sodium phosphate, 77 millimolar sodium chloride, and 0.01 percent weight per volume bovine serum albumin. We also prepared an acidic albumin solution, blending 24 millimolar sodium acetate, 79 millimolar acetic acid, and 0.1 percent weight per volume bovine serum albumin, adjusting its pH to 3.75 at 25 degrees Celsius. The enzyme solution was created using hyaluronidase (Sigma, H3884) at a stock concentration of 1,000 units per milliliter, prepared fresh in cold enzyme diluent, and then diluted further with cold enzyme diluent to yield a working solution of 6 units per milliliter for the reaction.

The assay procedure involved carefully pipetting precise volumes of reagents into suitable tubes to form a 2-milliliter reaction mixture, targeting final concentrations of 0.015 percent hyaluronic acid, 150 millimolar sodium phosphate, and 2 to 5 units of hyaluronidase. We began by mixing the contents and incubating the tubes at 37 degrees Celsius for 10 minutes. Additional reagents were then added, the mixture was gently swirled, and it was incubated at 37 degrees Celsius for exactly 45 minutes. After this period, 0.5 milliliters of each test and blank sample were transferred into cuvettes containing 2.5 milliliters of the acidic albumin solution, mixed immediately by inversion. The cuvettes were allowed to rest for 10 minutes at room temperature, with a critical note that any deviation in this incubation time could significantly alter the results. The percentage transmittance was measured at 600 nanometers using a spectrophotometer, calibrated against the blank. For valid results, the uncorrected percentage transmittance for each test needed to range between 130 and 170 percent, requiring a minimum of three valid readings per test to compute the activity. If necessary, the enzyme solution concentration was adjusted to keep results within this acceptable range.

The assay included a series of tests with varying combinations of Enzyme Solution and Enzyme Diluent. For Test 1, we combined 750 µL of Enzyme Solution with 250 µL of Enzyme Diluent. Test 2 featured 665 µL of Enzyme Solution and 335 µL of Enzyme Diluent. In Test 3, 585 µL of Enzyme Solution was mixed with 415 µL of Enzyme Diluent. Test 4 used equal parts, with 500 µL of each reagent. Test 5 consisted of 415 µL of Enzyme Solution and 585 µL of Enzyme Diluent, while Test 6 had 335 µL of Enzyme Solution and 665 µL of Enzyme Diluent. The blank sample contained no Enzyme Solution (0.00 milliliters) and 1.00 milliliter of Enzyme Diluent. Across all tests and the blank, 1.00 milliliter of Hyaluronic Acid Solution was consistently included, providing a uniform foundation for the samples. This carefully designed

setup enabled a thorough evaluation of enzyme activity under diverse conditions, with each test replicated in triplicate to reduce errors and enhance reliability.

To quantify the enzyme activity in units after completing the test, we analyzed the percentage transmittance values using a standardized formula. The unit of hyaluronidase activity was defined as the quantity of enzyme needed to induce a specific reduction in transmittance under the assay conditions. Specifically, one unit was determined as the amount of enzyme that decreases the percentage transmittance by an amount tied to the extinction coefficient, adjusted by the dilution factor and reaction volume. We relied on the Sigma-Aldrich determined extinction coefficient of 14.84, calculating the activity in units per milliliter as follows: the difference in percentage transmittance between the test and blank was multiplied by the dilution factor, then divided by the product of the extinction coefficient (14.84) and the reaction time (45 minutes). This methodical calculation ensured an accurate measure of the enzyme's capacity to degrade hyaluronic acid, reflecting its activity with precision under the specified conditions.

$$\text{Units/mL enzyme} = \frac{(\%T\ \text{Test} - \%T\ \text{Blank})\ (df)}{(14.84)\ (\text{ml of enzyme solution})} \qquad \text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

2.6.Phospholipase activity

To quantify the phospholipase A2 (PLA_2) activity in bee venom, a key enzymatic component contributing to its biological effects, we employed a colorimetric assay based on the Abcam protocol (ab133089). This assay measures the hydrolysis of a 1,2-dithio analogue of diheptanoyl phosphatidylcholine, which releases free thiols that react with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to produce a detectable color change. For each assay, we prepared a reaction mixture in a 96-well microplate by combining 10 µL of DTNB (10 mM in 0.4 M Tris-HCl, pH 8.0), 5 µL of assay buffer (25 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.3 mM Triton X-100), and 10 µL of diluted crude venom samples (1 mg/mL in ultrapure water). The reaction was initiated by adding 200 µL of the substrate solution, and absorbance was measured

at 414 nm—corresponding to the DTNB-thiol reaction product—every minute for 5 minutes using a microplate spectrophotometer (Dynex MRX). Reaction blanks, containing assay buffer and DTNB but no substrate, were included to account for background absorbance. All assays were conducted in triplicate to ensure reproducibility.

2.7.Cell culture and cytotoxicity

To evaluate the anticancer potential of bee venom, we tested its cytotoxicity against a panel of human cancer cell lines and a non-cancerous control. All cell lines were obtained from the National Repository Center (Iran) and included AGS (human gastric adenocarcinoma), A549 (human lung adenocarcinoma), HepG2 (human hepatocellular carcinoma), Panc-1 (human pancreatic carcinoma), DLCL-2 (human B-cell lymphoma), HT-29 (human colon adenocarcinoma), and normal human skin fibroblasts (HFF-1). We cultured the cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin, maintaining them at 37°C in a humidified incubator with 5% CO_2.

For the cytotoxicity assay, we employed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method to assess cell viability, following the protocol described (25). Each cell line, in its logarithmic growth phase, was seeded into 96-well plates at a density of 10^5 cells/well in 100 µL of DMEM and incubated for 24 hours to allow adhesion. The medium was then replaced with serum-free DMEM containing crude bee venom at concentrations of 0.5, 1, 2, or 5 µg/well. After an additional 24-hour incubation, we added 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) to each well and incubated the plates for 4 hours at 37°C. The medium was removed, and 150 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed by viable cells. The plates were gently shaken for 10 minutes, and the absorbance of the formazan product was measured at 570 nm using a microplate reader (Dynex MRX). Untreated cells served as a negative control, while DMSO alone was used as a vehicle control. All assays were performed in triplicate to ensure reproducibility.

2.8 Statistical Analysis

All assays (including melittin quantification, PLA_2 activity, hyaluronidase activity, hemolytic activity in Table 1, and MTT cytotoxicity assays in Figure 4) were performed in triplicate, and

results are expressed as mean ± standard deviation (SD). Seasonal comparisons within each province were conducted using paired Student's t-tests. Provincial differences within each season were analyzed by one-way ANOVA followed by Tukey's post-hoc test. For cytotoxicity data (CC_{50} values in Figure 4), two-way ANOVA (factors: season and province) with Sidák's multiple comparisons test was applied. Statistical significance was defined as $p < 0.05$. All analyses were performed using GraphPad Prism version 9.0.

3.Results

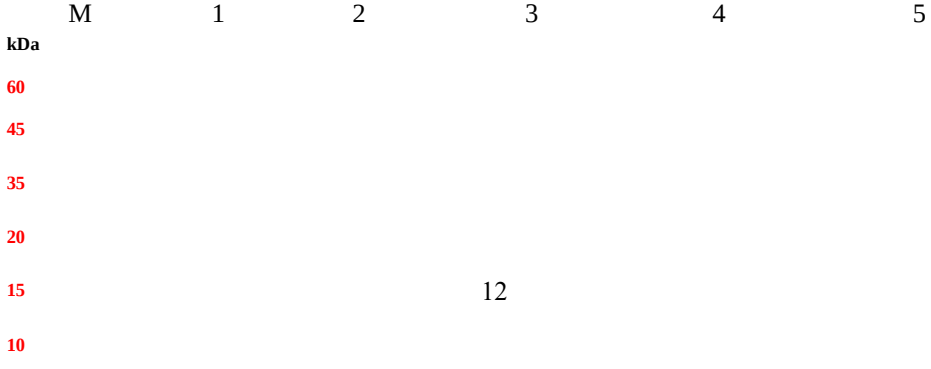
3.1.SDS PAGE

To investigate how Iran's diverse climates and seasons influence bee venom's protein composition, we conducted sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on both crude and purified venom samples from five provinces: Alborz (semi-arid), Ardabil (cold and mountainous), Fars (warm and wet), Hormozgan (warm and dry), and Mazandaran (moderate and humid), collected in spring and autumn of 2023. Using SDS-PAGE under non-reducing conditions, we separated proteins by molecular weight to compare crude venom profiles and confirm the purity of isolated melittin, the venom's primary peptide, laying the foundation for its therapeutic evaluation. As shown in Figure 1A, the 12% polyacrylamide gel of crude venom revealed a consistent pattern of protein bands ranging from 2 to 30 kDa across all samples. A sharp, prominent band at approximately 2.8 kDa, corresponding to melittin (59.25–72.85% of dry weight, Table 1), dominated every lane, highlighting its stability across regions and seasons. This consistency underscores melittin's potential as a reliable anticancer agent. Other bands, likely representing phospholipase A2 (~19 kDa), were visible, though higher-molecular-weight proteins like hyaluronidase (~38 kDa) were less prominent in this range. We stained the gel with Coomassie Brilliant Blue R-250, ensuring clear visualization of low-molecular-weight peptides.

Figure 1B displays the SDS-PAGE analysis of purified melittin, isolated via semi-preparative HPLC (Section 3.2), from samples across the five provinces and additional regions. The 12% polyacrylamide gel, optimized for low-molecular-weight peptides, showed a single, sharp band at ~2.8 kDa in each lane, confirming the high purity of the isolated melittin. The lanes, representing both spring and autumn samples where available, demonstrated melittin's consistent molecular weight, aligning with mass spectrometry data (supplemetray files) that verified its structural integrity.

The gels, with lanes arranged after molecular weight markers, revealed highly similar profiles for crude venom (Figure 1A) and uniform melittin bands for purified samples (Figure 1B). This suggests a conserved venom composition, with minor variations in crude venom band intensity reflecting regional and seasonal differences, such as Fars' 20% melittin variation (Table 1).

A



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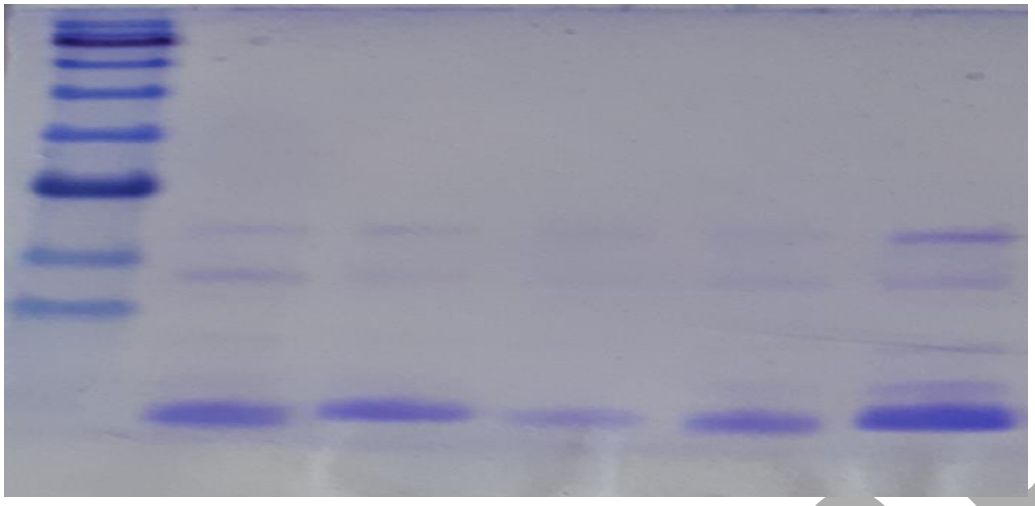


B

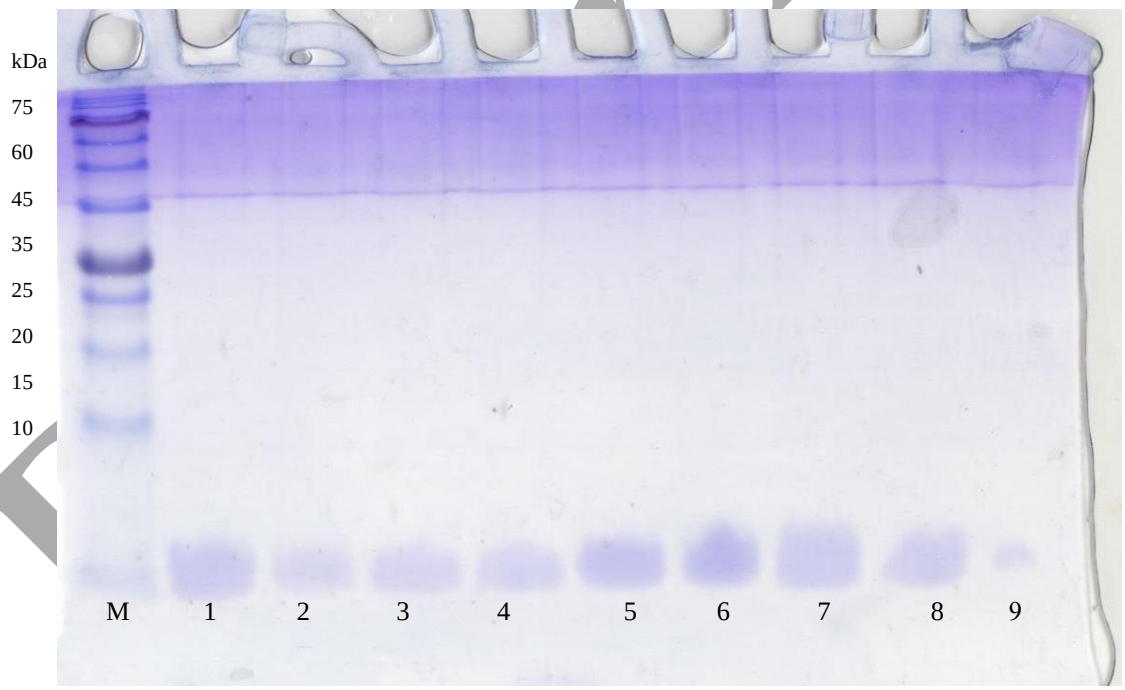


Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude and purified bee venom from Iranian provinces. (A) Crude venom samples (10 µg per lane) from spring and autumn 2023, separated on a 12% polyacrylamide gel under non-reducing conditions at 120 V for 90 minutes and stained with Coomassie Brilliant Blue R-250.

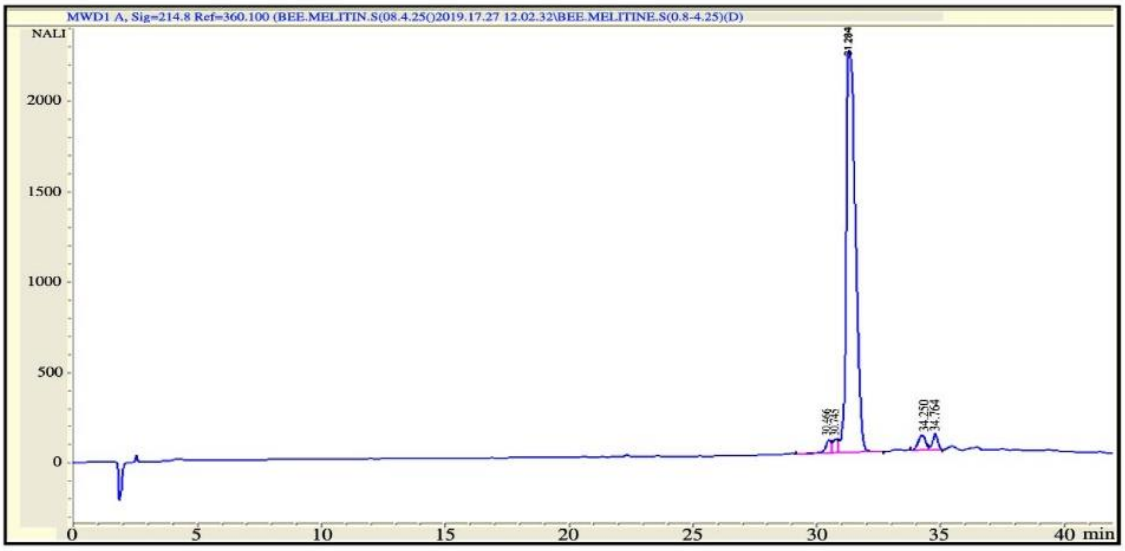
Lanes, from left to right: molecular weight marker (2–250 kDa); (1) Alborz; (2) Ardabil; (3) Fars; (4) Hormozgan; (5) Mazandaran. A prominent melittin band (~2.8 kDa) dominates the 2–30 kDa range, indicating consistent venom composition across regions and seasons. (B) Purified melittin samples (5 µg per lane), isolated via semi-preparative HPLC, separated on a 12% polyacrylamide gel under non-reducing conditions and stained with Coomassie Brilliant Blue R-250. Lanes, from left to right: molecular weight marker (2–250 kDa); (1) Fars (autumn); (2) Fars (spring); (3) Gilan; (4) Ardabil (spring); (5) Ardabil (autumn); (6) Hormozgan (spring); (7) Mazandaran (autumn); (8) Mazandaran (spring); (9) Alborz (spring). A single melittin band (~2.8 kDa) confirms the purity and consistent molecular weight across regions and seasons.

3.2.HPLC and melittin purification

The chromatogram of all collected bee venoms from the stated provinces were prapared through a C18 column and their amout of % melittin was summerised in table 1(chromatograms are not shown here) . As the figure 2 shows the running method was checked by a commerial pure melittin that had a retention time of elution at 30 minutes. Nearly in all chromatograms there were around 15 main peaks while their amount of melittins were varied. All crude venoms were fractionated for their pure melittin through a semi prepar HPLC. The melittin fraction at the retention time 60-70 minutes was gathered and after drying it in a freeze dryer controled by analytical HPLC (figure 3).Result revealed the method was efficient in semiprep method to have large amountof pure melittin. The preparation was applied to all crude venoms from the provinces with same method (data are not shown here).The pure peptide was stored at -20˚C for future experments. The pure melittin samples were analysed for their mass spectrum. Comparing the mass spectromertry data that are shown here, spectrum in pure melittin samples from this study was in agreement with the one of standard melittin and other studies(8).

The chromatograms of all collected bee venoms from the specified provinces were prepared using a C18 column, and the percentage of melittin was summarized in Table 1. Figure 2 demonstrates that the running method was validated using a commercially pure melittin with a retention time of 30 minutes. In nearly all chromatograms, approximately 15 main peaks were observed, each with varying amounts of melittin. All crude venoms were fractionated to isolate pure melittin using semi-preparative HPLC. The melittin fraction eluting at 60-70 minutes was collected and subsequently dried using a freeze dryer, as depicted in figure 3 and confirmed by

analytical HPLC. Results indicated that the semi-preparative method was efficient in obtaining a substantial amount of pure melittin. This process was applied to all crude venoms from the provinces using the same method. pure HPLC grade melittin samples was assessed for PLA2 and hyaloronidase activity where there was no detectable enzymes activities in theses samples according to our methodology.The pure peptide was stored at -20˚C for future experiments. The pure melittin samples underwent mass spectrum analysis. Pure melittin samples used in the research project collecting from fall and spring seasons were examined along with the mass spectrum of the standard sample that was obtained from the Sigma firm. All spectra from the standard melittin and purified sample display characteristic peaks corresponding to the quadruply protonated ion [M+4H]^{4+} at m/z 714 (serving as the precursor and often the base peak in MS1), the triply protonated ion [M+3H]^{3+} at m/z 951-952, and the doubly protonated ion [M+2H]^{2+} at m/z 1426, with chromatograms showing sharp elution peaks at similar retention times around 1.45-1.65 min, consistent with the additional sample data in the uploaded image (though specific data for all samples are not shown). The MS1 data confirm matching molecular masses, while the MS2 fragmentation patterns of the m/z 714 precursor align closely across both samples and the additional image, featuring a prominent base peak at m/z 813 and shared major fragments (e.g., 129, 170, 186, 228, 542, 617, 672, 728, 804, 811, 812, 815, 895, 991, 1056), indicative of identical peptide sequence cleavages typical for melittin. Minor differences in low-abundance peaks or intensities likely stem from sample purity variations or matrix effects in the bee venom extract, but no mass shifts suggest structural variants. Consequently, the standard and purified melittin from the study share the same sequence, supporting uniformity across local bee venom sources (supplementary files).



401

402 Figure_2 Reverse-phase HPLC separation of venom proteins from honey bee venom . The
403 chromatogram for pure melittin from sigma. C18 (100Å, 5 µm, 4.6 × 150 mm) was used
404 through two solvent A (water, 0.1% TFA) and B (80% acetonitrile, 0.1% TFA) in a linear
405 gradient at a flow rate of 1 ml/min for 40 minutes. The fractions were monitored at 214 nm
406 wavelength. Melittin was eluted at 30 min at 40% of acetonitrile. The explained method was
407 run for all other bee venom samples that were collected from mentioned provinces.

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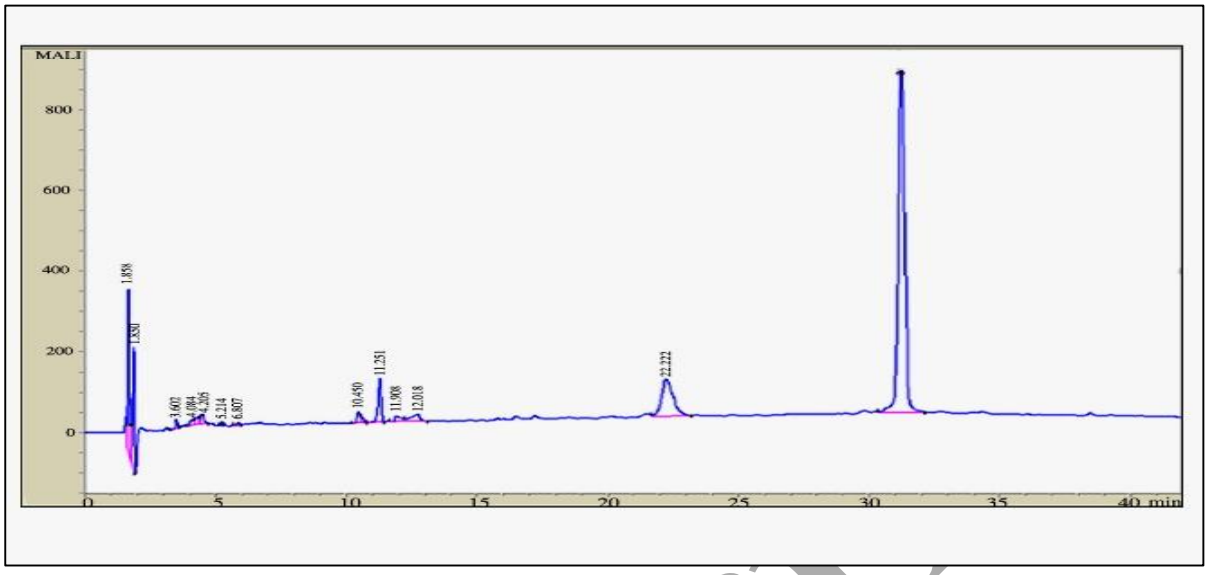
MALI
800
600
400
200
0
1.858
1.930
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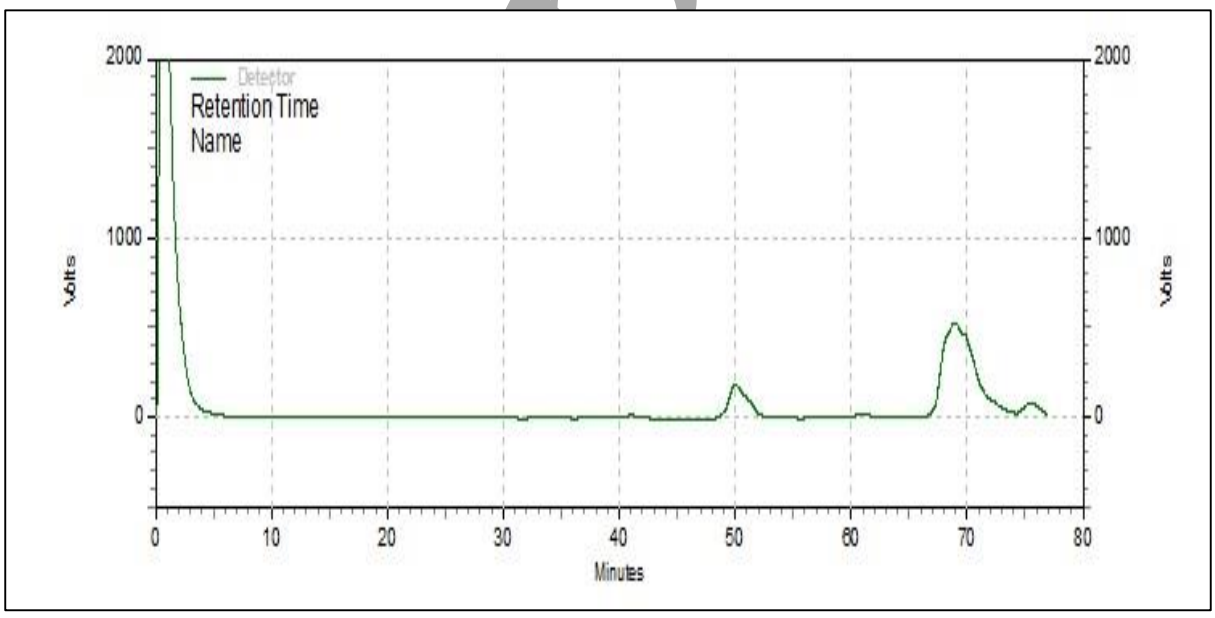
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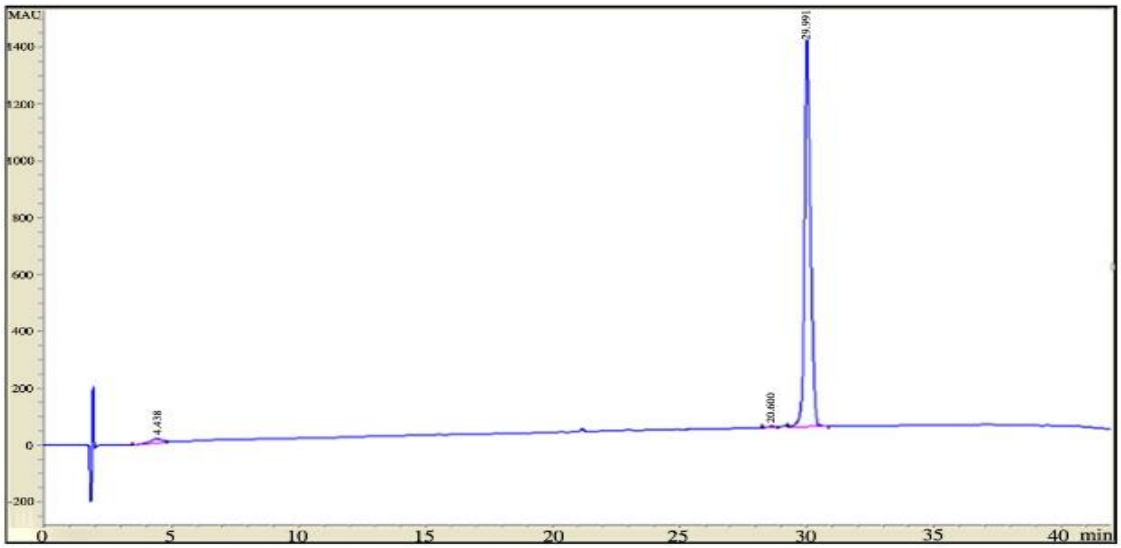

Figure_3 semi-prep HPLC to purification of melittin. The analytical HPLC chromatogram of crude venom of Mazandaran province spring sample(A).The chromatogram of a preparative procedure to purify large amount of melittin form Mazandaran province spring sample, shows a retention time of 65-70 minutes that was collected (B). The pure collected and lyophilized fraction of preparative procedure checked for its impurity as explained earlir through analytical HPLC (C).

3.3.Venom characterization from the collection

To unravel the biochemical diversity of Iranian bee venom and its potential as a cancer therapy, we analyzed melittin content, phospholipase A2 (PLA_2) activity, hyaluronidase activity, and hemolytic activity in crude venom samples collected from five provinces—Alborz (semi-arid), Ardabil (cold and mountainous), Fars (warm and wet), Hormozgan (warm and dry), and Mazandaran (moderate and humid)—during spring and autumn 2023. These properties, summarized in Table 1, were quantified using high-performance liquid chromatography (HPLC) for melittin, kinetic assays for PLA_2, turbidometric assays for hyaluronidase, and hemolytic assays for red blood cell lysis, all conducted in triplicate for robust results (Sections

2.3–2.6). This detailed characterization illuminates how Iran's varied climates and seasons shape venom's potency, offering a foundation for tailored therapeutic applications.

Melittin, the primary peptide driving venom's anticancer effects, ranged from 59.25% to 72.85% of venom dry weight across all samples, as determined by HPLC (Table 1). The highest melittin content was found in Hormozgan's autumn sample (72.85%), likely influenced by its warm, arid climate, which may enhance peptide synthesis. In contrast, Mazandaran and Ardabil maintained stable melittin levels around 59% in both seasons, suggesting that cooler or humid conditions foster consistency. Alborz's spring sample showed approximately 10% higher melittin than its autumn counterpart, while Fars exhibited the largest seasonal variation, with a 20% difference between spring and autumn. These findings highlight how warmer regions and seasons amplify melittin production, a critical factor for its cytotoxicity against cancer cells (Figure 4).

Researching into enzyme activity, PLA_2 levels, measured via the Abcam kinetic assay (ab133089), peaked in Fars' spring sample but dropped by approximately 60% in autumn, reflecting significant seasonal fluctuations in this warm, wet region. Other provinces showed relatively stable PLA_2 activity between seasons, indicating that extreme climates, such as Ardabil's cold or Mazandaran's humidity, may stabilize enzyme expression. Hyaluronidase activity, assessed turbidometrically, reached its highest level in Fars' autumn sample (101.66 units), while other provinces averaged around 80 units in both seasons, suggesting limited variability except in warmer, wetter conditions. Hemolytic activity, ranging from 0.92 to 1.71 µg/mL across all samples, showed no significant regional or seasonal differences, indicating a consistent baseline toxicity profile suitable for therapeutic evaluation.

These results, drawn from Table 1, reveal nuanced variations in venom composition driven by Iran's diverse climates and seasons. The elevated melittin and enzyme activities in autumn samples from warmer regions like Hormozgan and Fars correlate with their superior anticancer effects, particularly against lymphoma, gastric, and pancreatic cell lines (Section 3.4, Figure 4). By mapping these biochemical differences, our findings pave the way for optimizing venom extraction strategies and developing targeted cancer therapies, harnessing Iran's rich environmental diversity to enhance bee venom's therapeutic potential.

Table 1 Analysis of honey bee venom for melittin and biological activities collected from five provinces of Iran with varying climates.

	Melittin % [a]		**PlA2 activity [b] (U/ml)**		**Hyaloronidase [c](unit/50 µg venom)**		**Hemolytic activity [d] (µg/ml)**	
Province	Autumn	Spring	Autumn	Spring	Autumn	Spring	Spring	**Automn**
Ardabil	59.91±0.053	58.53±0.259	0.49±0.009	0.42±0.016	79.33±3.29	86.66±6.23	1.25±0.021	**0.92±0.052**
Mazandaran	59.84±0.251	60.41±0.016	0.28±0.012	0.24±0.004	74.00±1.41	78.66±1.88	1.71±0.062	**1.29±0.032**
Alborz	59.25±0.032	50.61±0.021	0.40±0.008	0.49±0.008	80.00±4.08	86.66±6.23	0.89±0.20	**0.98±0.023**
Fars	63.85±0.057	43.23±0.024	0.63±0.024	0.22±0.016	101.66±6.23	71.66±2.35	1.19±0.032	**1.1±0.081**
Hormozgan	72.85±0.040	42.44±0.129	0.28±0.009	0.24±0.007	83.33±2.35	81.66±9.42	1.61±0.008	**1.0±0.013**

a; melittin% was calculated from HPLC chromatogarams for each sample in three round of experiment, b; PLA2 was assayed by the kinetical method, Abcam ab133089 in triplicate for each sample, c;hyaloronidase was calculated by turbidometric assay in triplicate for each venom, d; hemolytic assay was carried out in triplicate for each sample .

3.4.cytotoxicity effect of bee venom

To explore the anticancer potential of Iranian bee venom, we evaluated its cytotoxicity against a panel of human cancer cell lines—AGS (gastric adenocarcinoma), A549 (lung adenocarcinoma), HepG2 (hepatocellular carcinoma), Panc-1 (pancreatic carcinoma), DLCL-2 (B-cell lymphoma), and HT-29 (colon adenocarcinoma)—using crude venom samples from five provinces (Alborz, Ardabil, Fars, Hormozgan, Mazandaran) collected in spring and autumn 2023. Employing the MTT assay to measure cell viability (Section 2.7), we tested venom concentrations ranging from 0.5 to 5 µg/well over 24 hours, with results expressed as CC50 values (the concentration reducing cell viability by 50%) in Figure 4. These findings highlight venom's potency and its variation across regions and seasons, offering insights into its therapeutic promise.

As shown in Figure 4, autumn venom samples consistently outperformed spring samples in cytotoxicity. For HT-29 cells (Figure 4A), all autumn venoms achieved CC50 values below 2 µg/well, indicating high potency, while spring samples exceeded 2 µg/well, except for Hormozgan's, which remained highly effective. DLCL-2 cells (Figure 4B) were particularly sensitive to autumn venoms, with CC50 values below 1 µg/well across all provinces, compared to spring samples, where only Hormozgan's venom matched this potency. Panc-1 cells (Figure 4C) showed CC50 values below 2 µg/well for autumn venoms, with Hormozgan and Fars exhibiting superior potency in both seasons, while spring samples required higher concentrations. HepG2 cells (Figure 4D) responded to autumn venoms with CC50 values below 2 µg/well, except for Alborz's, which aligned with spring samples from Mazandaran, Alborz, and Hormozgan (around 2 µg/well). A549 cells (Figure 4E) were less sensitive, with CC50 values around 2 µg/well for both seasons across most provinces, though Alborz's spring sample required over 4 µg/well, indicating lower efficacy. AGS cells (Figure 4F) were highly responsive to autumn venoms, with CC50 values at or below 1 µg/well, while spring samples from Ardabil, Mazandaran, and Fars required approximately 2.5 µg/well, reflecting seasonal variability.

These results, detailed in Figure 4, reveal that autumn venoms, particularly from warmer regions like Hormozgan and Fars, exhibit enhanced cytotoxicity, likely due to higher melittin content (e.g., 72.85% in Hormozgan's autumn sample; Table 1). DLCL-2, AGS, and Panc-1 cell lines proved the most sensitive, with autumn CC50 values consistently below 1–2 µg/well, suggesting their suitability as targets for venom-based therapies. In contrast, A549 and HepG2

cells showed greater resistance, requiring higher concentrations. The lack of cytotoxicity data for the non-cancerous control (HFF-1; Section 2.7) limits conclusions about selectivity, but the observed potency aligns with melittin's membrane-disrupting properties (Section 3.3). These findings underscore Iranian bee venom's potential as a natural anticancer agent, with seasonal and regional variations—particularly autumn's superior efficacy—guiding future therapeutic development and extraction strategies.

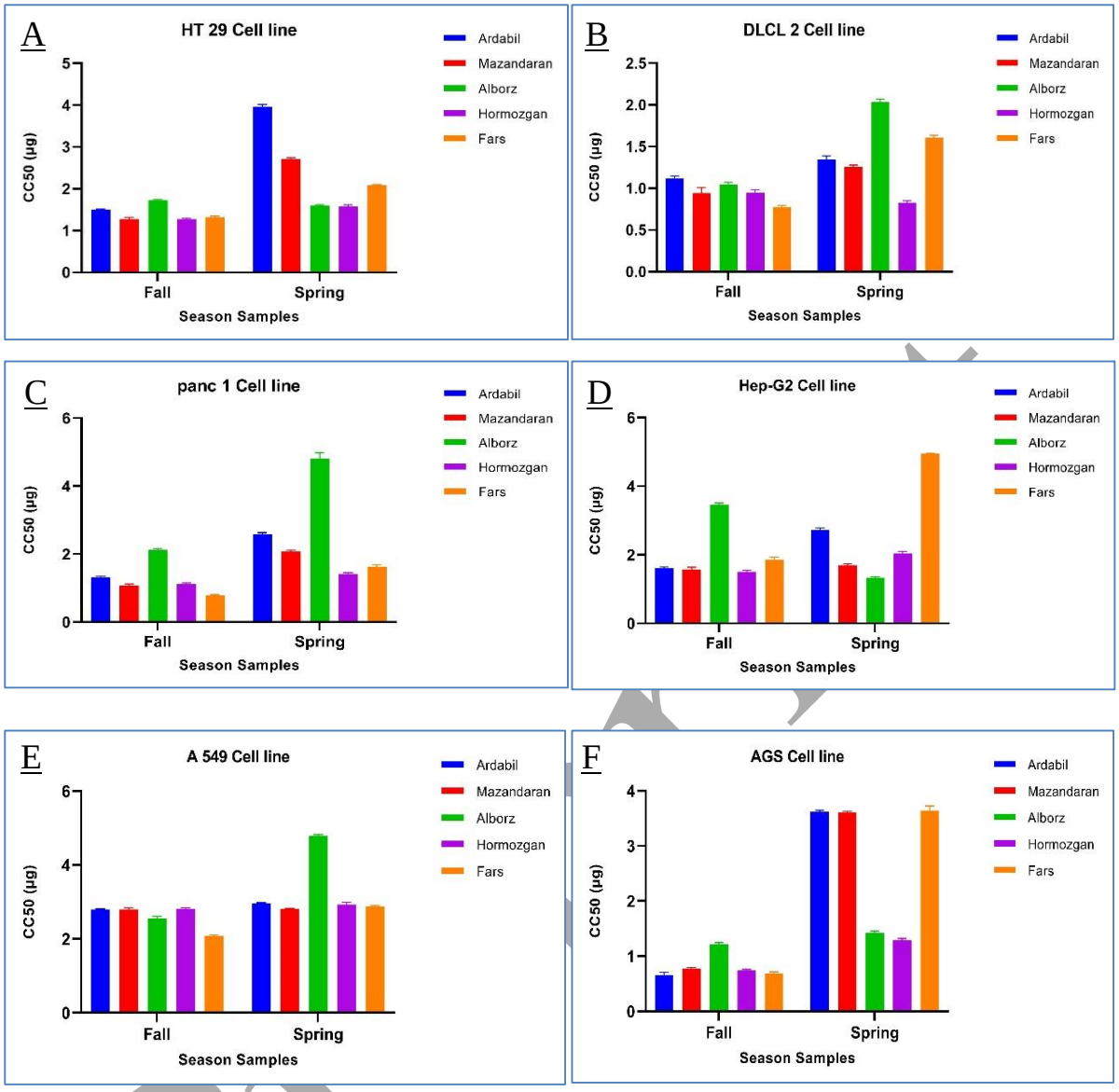

Figure 4: Cytotoxicity of crude bee venom from five Iranian provinces (Alborz, Ardabil, Fars, Hormozgan, Mazandaran), tested via MTT assay on human cancer cell lines. Panels A–F show CC50 values (µg/well, concentration reducing cell viability by 50%) for spring and autumn 2023 samples (0.5–5 µg/well, 24 h): A) HT-29 (colon adenocarcinoma), B) DLCL-2 (B-cell lymphoma), C) Panc-1 (pancreatic carcinoma), D) HepG2 (hepatocellular carcinoma), E) A549 (lung adenocarcinoma), F) AGS (gastric adenocarcinoma). Autumn venoms from warmer regions (Hormozgan, Fars) achieve CC50 values below 1–2 µg/well, showing enhanced potency for DLCL-2, AGS, and Panc-1, linked to higher melittin content (Table 1), highlighting bee venom's anticancer potential.

4.Discussion

Bee venom of *Apis mellifera* is a complex mixture containing melittin, phospholipase A_2, hyaluronidase, histamine, catecholamines, and serotonin, all contributing to its biological activity (2,6,26). These components act synergistically to produce antimicrobial, inflammatory, and cytotoxic effects (1,3). Among them, melittin represents the major peptide fraction and is primarily responsible for the venom's therapeutic and anticancer properties (4,5). Research has shown that bee venom has radioprotective and antimutagenic properties (27,28), as well as anti-inflammatory and pain-relieving effects (29,30). Additionally, recent studies have documented its impact on apoptosis, necrosis, cell proliferation, cytotoxicity, and the inhibition of certain cancer cell types (31). The protein content of bee venom is considerably influenced by numerous factors such as age, strain, social position, geographic location, season, and climate (7,8). Temperature has an extremely significant effect on the venom's protein profile and biological activity (9). Several studies, including Iranian studies, have revealed a wide array of pharmacological activities such as immunomodulatory, anti-inflammatory, analgesic, antiapoptotic, and antiarthritic activities (10–14). Primarily, the anticancer potential of bee venom, through melittin's ability to lyse cancer cell membranes and induce apoptosis, has been of widespread interest (15,16). Melittin's potential for selective targeting of cancer cells renders it a promising candidate for novel cancer treatments (5,17). Advancements in nanotechnology have also improved targeted delivery of melittin (22). According to recent meteorological research (32), the selected provinces represent distinct climatic zones: Alborz (cold semi-arid high plateau), Ardabil (cold mountainous semi-arid), Fars (hot semi-arid), Hormozgan (hot desert), and Mazandaran (humid subtropical). Ecological factors such as nectar quantity, temperature, and humidity were considered (21). Intraspecific variation, age, colony strength, defense behaviour, nutrition, season, and collection methods further affect venom profiles (33). Protein compound variations across bee strains and tasks also contribute to this diversity (34,35).

Melittin content ranged from 59.25% to 72.85%, with the highest value (72.85%) in autumn samples from Hormozgan (hot desert). These values are among the highest reported globally and exceed the typical 40–60% range (36). Comparable high levels have been documented in Polish autumn collections (up to 70%) (44), Portuguese venoms (50–60%) (45), and certain Romanian spring samples (up to 86%) (46), whereas Turkish bees showed only ~46% (46) and

earlier Iranian studies reported 50–70% (19,20). Higher temperatures (>30°C) and low humidity in Hormozgan and Fars during autumn prolong foraging and stimulate venom-gland metabolism, resulting in ~20% seasonal increases in melittin and elevated PLA_2 and hyaluronidase activities (7–9,33). In contrast, colder provinces (Alborz, Ardabil) exhibited stable profiles, consistent with limited foraging and lower metabolic rates under cooler conditions (20,21). These patterns reinforce previous observations in Africanized (9) and Aegean ecotypes (8), where temperature thresholds drive peptide upregulation, and extend Iranian regional data (19,20) by demonstrating pronounced climatic gradients within a single country (32).

Crude venom exhibited potent cytotoxicity (CC_{50} frequently <1–2 µg/well in autumn samples from warmer provinces) against DLCL-2 (lymphoma), AGS (gastric), Panc-1 (pancreatic), HT-29 (colon), HepG2 (liver), and A549 (lung) cell lines. Direct comparisons with studies that also used crude bee venom on the same cell lines show that Iranian material ranks among the most active reported: AGS: $CC_{50} \leq 1$ µg/well vs. ~3–5 µg/mL (Korean crude venom) (43) and ~5–10 µg/mL (Portuguese crude venom) (45). Panc-1: comparable or superior to Zhao et al. (2022) (~2–4 µg/mL) (40). HT-29: comparable to Duarte et al. (2022) using crude venom alone (<2 µg/mL) (37). HepG2: aligns with crude-venom-inclusive studies (~2 µg/mL) (41). DLCL-2: first report with crude venom; previous lymphoma work used marine compounds or adjuncts (39). A549: required higher concentrations, consistent with multi-mechanistic reports (42). The superior potency correlates directly with higher melittin and enzyme levels, confirming synergistic action of the whole venom matrix (3,4,5,26,38).

This study provides valuable insights into the climatic and seasonal influences on Iranian bee venom composition and its promising in vitro anticancer activity, while opening exciting avenues for further research. As a primarily descriptive work, it lays a solid foundation by characterizing venom variations and cytotoxicity, though the assays do not yet include detailed molecular mechanistic investigations (e.g., specific pathways of apoptosis, cell cycle arrest, or necrosis induced by melittin and other components). The evaluations were conducted in vitro, offering strong preliminary evidence of potency and paving the way for future in vivo studies to evaluate efficacy, selectivity, and safety in animal models. Additionally, the inclusion of genotyping local *Apis mellifera* populations and assessing factors such as colony nutrition, health, and gut microbiota promises to further enrich our understanding of venom production.

Genotyping native Iranian bees to link genetic variation with venom potency represents a highly promising direction. Similarly, investigating honey bee gut microbiota, nutrition, and colony health as potential modulators of venom bioactivity offers great potential. Ultimately, in vivo studies and clinical trials will be essential to advance these encouraging results toward real-world therapeutic applications.

5.Conclusion

Iranian bee venom shows remarkable climatic and seasonal variation, with melittin reaching 72.85% in autumn samples from hot-arid Hormozgan—one of the highest values worldwide. Warm-region autumn venom exhibits significantly higher phospholipase A_2 and hyaluronidase activities and superior cytotoxicity (CC_{50} <1–2 µg/well) against lymphoma (DLCL-2), gastric (AGS), and pancreatic (Panc-1) cancer cells. Temperature, humidity, and foraging-season length are the primary drivers of this enhanced potency. Selective autumn collection from hot provinces therefore provides a simple, cost-effective strategy to obtain highly potent crude bee venom for anticancer applications, highlighting the exceptional therapeutic potential of regionally optimised Iranian bee venom.

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Authors' contributions

Conceptualization, A.N. and F.T., M.SA.; resources and supervision, A.N. and M.M.; methodology, F.T., M.SA., M.SH., M.M., A.N.; Data interpretation and analysis and investigation, A.N. , F.T., M.SA.; data curation, A.N. and F.T., M.SA.; writing original draft preparation, A.N., M.SH.; writing review and editing. All the authors revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of interests

The authors declare no conflict of interest.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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