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INHIBITION PROPERTIES OF CLINICALLY IMPORTANT ENZYMES IN WOUND HEALING BY BEE VENOM

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ABSTRACT

Apitherapy has importance in traditional and complementary medicine practices, including applications made by directly or functionalizing honey, pollen, propolis, bee venom, and bee bread (perga) obtained from honey bee colonies. Bee venom is preferred by apitherapists and is directly applied to the body from the bee sting or used in crystalline form. Apitoxin is a product that is formed in the venom bag of bees and contains mainly apamin, melittin, MCD-peptide, hyaluronidase, histamine, and phospholipase A2. Due to its these active ingredients, bee venom is effective in arthritis, central and peripheral nervous system diseases, respiratory diseases, gastroenterology, cardiovascular system diseases, eye diseases, skin diseases, urology, endocrinology, cancer diseases, and using it in the cosmetics industry is very popular. Although bee venom is widely used directly or indirectly in apitherapy applications, it is not possible to use standardized bee venom as a drug. In this study, the amount of total protein, melittin, and phospholipase A2 amounts of bee venom samples were determined and their inhibition effects on collagenase and myeloperoxidase enzymes were examined. The mean amounts of phospholipase A2, apamin, and melittin contained in bee venom samples were found to be 46.38%±0.10%, 2.19±0.11%, and 12.46±0.19%, respectively. It was determined that bee venom samples inhibited the collagenase enzyme by more than 50%. It shows that bee venom has an inhibitory effect on clinically important enzymes. However, it is necessary to conduct allergy tests before the use of the bee venom directly.

Keywords: *Apis mellifera* L., apitoxin, collagenase, myeloperoxidase, wound healing.

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INTRODUCTION

The honey bee (*Apis mellifera* L.) is a social insect and it is important for the pollination of plants, it is also used for economic benefits of bee products such as pollen, honey, beeswax, propolis, bee bread, bee venom and royal jelly (Porrini *et al*., 2003). They are widely used to treat illnesses such as central and peripheral nervous system diseases, cardiovascular system diseases, eye diseases, skin diseases, urology, gastroenterology, endocrinology, and cancer diseases. This treatment method is called apitherapy which bee products are used in traditional and complementary medicine applications (Shimpi *et al*., 2016; Saleh, 2017; Sig *et al*., 2019).

Bee venom, also known as apitoxin, is the most preferred bee product in apitherapy applications. It is a mixture of acidic and basic secretions produced in the venom glands in the abdominal cavity of worker bees (Figure 1) (Bogdanov, 2015; Shimpi *et al*., 2016). It is yellowish in colour, bittersweet, pungent, normally in liquid form but it dries and crystallizes after contact with air (Bogdanov, 2015; Altıntaş and Bektaş, 2019). The amount of venom in a bee varies according to the region, season, and breed of the bee (Altıntaş and Bektaş, 2019). A honey bee delivers between 0.15 and 0.3 mg of venom on average and one gram of dry powdered venom can be collected from 20 hives in two hours (Figure 2) (Çaprazlı and Kekeçoğlu, 2021). Bee venom consists of a complex structure containing many substances such as 88% water and the remaining 12% peptides, phospholipids, bioactive amines, amino acids, sugars, pheromones, enzymes, and minerals (Shimpi *et al*., 2016; Gülmez *et al*., 2017; Sig *et al*., 2019). However, the World Health Organization's Allergen Nomenclature Sub-Committee (WHO/IUIS) reported the 12 protein fractions of bee venom as allergenic (Burzyńska and Piasecka-Kwiatkowska, 2021) so using bee venom is limited and the allergy tests should be performed before the use. Bee venom is seen as a natural medicine with many pharmacologically effective elements in its structure (Kurek-Górecka *et al*., 2021). It is known for its many biological activities such as antibacterial, fungicide, antiviral, anti-inflammatory, anti-arthritis, and anticancer (Bogdanov, 2015; Shimpi *et al*., 2016; Gülmez *et al*., 2017; Saleh, 2017; Altıntaş and Bektaş, 2019).

A wound is a disruption of tissue integrity due to a cut, burn, or disease. Different criteria are used to classify wounds and tissue layers are widely used method in classification. The skin is composed of epidermis, dermis, and hypodermis. If the affected skin layer is the epidermis, the wounds are classified as superficial, if the dermis and partial blood vessels are involved in the injury, then they are classified as thick wounds. The healing process is a series of events in which many successive cell types interact with each other (Schultz *et al*., 2000; Abdelrahman and Newton, 2011). This process is divided into 5 steps: hemostasis, inflammation, migration, proliferation, and maturation (Schultz *et al*., 2000; Abdelrahman and Newton, 2011). Healing of skin wounds occurs through complex and gradual cellular functions. These functions are interrelated and each function can be controlled by certain local or systematic factors. Although the healing of small wounds can be controlled, while, it is very difficult to control the healing of diabetes, cancer, chronic, infected, or large wounds. The wound healing rate of patients with such chronic diseases is quite slow (Frykberk and Banks, 2015) and their wounds show different complications. Therefore, wound healing management is important and bee venom is a promising bee product due to its anti-inflammatory effect and wound-repairing capacity (Kurek-Górecka *et al*., 2021). The chronic wound is a complicated matrix characterized by the simultaneous interaction of elevated levels of neutrophils, neutrophil-derived proteolytic enzymes, and reactive oxygen species (Díaz-GonzáLez *et al*., 2012). Matrix metalloproteinase, an endopeptidases, catalyzes the formation of the compounds of the extra-cellular matrix such as elastin and collagen, and growth factors formed in the wound site, thereby impairing the healing (Díaz-GonzáLez *et al*., 2012).

Collagen is largely present in chronic wounds. It is an integral structural compound of the extracellular matrix and all connective tissues, is active in all stages of the wound healing process (Gelse *et al*., 2003). It is associated to human health and aging-related disorders (Baumann, 2007). Collagen degradation in human begins with the activation of a proteolytic enzyme called collagenase is a member of the matrix metalloproteinase enzymes (Holmbeck & Birkedal-Hansen, 2013). Many studies on the prevention of skin aging have been done on antioxidant activities with the inhibition of collagenase synthesis and the increase in collagen production (Ganceviciene *et al*., 2012; Ghimeray *et al*., 2015; Panichakul *et al*., 2022; Flores de los Rios *et al*., 2023). Myeloperoxidase (MPO) is another important enzyme in wound healing (Tay and Tamam, 2013). Macrophages play a role in the destruction of the collagen layer in the atheroma plate through the secretion of matrix metalloproteinase enzymes and metal-independent myeloperoxidase. Macrophages participate in the occurrence of acute coronary events by weakening the fibrous capsule (Mullane *et al*., 1975; Apple *et al*., 2005).

Therefore, inhibition of both MPO and collagenase enzymes is important in wound healing (Ågren *et al*., 1992; Galijašević, 2021). In this study, the inhibition effect of whole bee venom directly on collagenase and myeloperoxidase was performed for the first time. The amount of total protein, melittin, phospholipase A2, and apamine of bee venom obtained from different hives were determined and compared to determine whether bee venoms obtained from different origins had different biological activities or not.

MATERIALS AND METHODS

Bee venom samples were purchased from five different apiaries located in different districts of Malatya (Yeşilyurt, Battalgazi, Akçadağ, Arapkir, and Kale) Turkey, by good beekeeping practices (Figure 3, Table 1). The collected venom samples were stored at -20 ^{0}C in an amber bottle. Since animals were not used directly in this study, ethical permission was not required.

Bee venom was dissolved in 0.9% NaCl solution for improving the solubility of proteins (salting in). The amount of total protein was determined according to the Bradford (1976). In this technique, when the negatively charged Coomassie Brilliant Blue G 250 reagent binds to positively charged groups in the protein, the colour of the solution turns blue colour and absorbs at 590 nm by UV absorption spectroscopy (Hach, DR/4000U). The determination was made by preparing a curve of absorbance versus concentration using bovine serum albumin (BSA) as a standard. Analyses were made in three replicate and the main value was calculated.

To determine the biological activities and chemical content of bee venom samples, bee venom water extract (5mg of bee venom/ 10mL distilled water) was prepared for each sample, separately. Apamin, phospholipase A2, and melittin analyses were performed on a UV detector High Pressure Liquid Chromatography (HPLC, Hitachi-VWR) device at 220 nm with a Supelco Supelcosil LC-318 column. For mobile phase A, ultrapure water and 0.1% trifluoroacetic acid; for mobile phase B 80:20 acetonitrile: ultrapure water, 0.1% trifluoroacetic acid were used. A linear gradient method was applied and 5-80% mobile phase B was used. The flow rate is 1 ml/min and the injection volume is 40 µl (Samancı and Kekeçoglu, 2019). Standard solutions were formed as 2%, 5%, 10%, 20%, 40%, and 50% by taking the appropriate amount from each solution and analysed with HPLC to obtain calibration curves. Three replicates were analysed for each sample.

Inhibition of collagenase activity was determined by monitoring the segmedation of the specific collagenase substrate N-[3-(2-Furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA) using the enzyme

pre-incubated with different amount of bee venom (2-10µL). Collagenase enzyme was mixed with 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $CaCl_2$ and 400 mM NaCl for 15 min at room temperature. Then, 0.8 mM FALGPA was injected to the bee venom mixture and incubated at 37 °C for 20 minutes. The distilled water instead of the enzyme was used for the blank tube. The absorbance was recorded at 340nm at the end of the incubation (Tu and Tawata, 2015). Collagenase inhibition was calculated according to the formula below (Khalil *et al*., 2008).

To determine the inhibitory effect of bee venom on myeloperoxidase (MPO), 2.5 nM enzyme was incubated with 50 mM phosphate buffer (pH 7.4) containing 0.5 mM H_2O_2 and bee venom at different amounts for 15 minutes. Then, 1 mM guaicol (substrate) solution was added and an increase in absorbance was measured at 470 nm in 3 minutes at 37 ºC. MPO inhibition was calculated according to the formula below (Khalil *et al*., 2008).

% Inhibition = [1- (inhibitory activity/Control activity)]x 100

All chemicals used in analyses were purchased from Sigma-Aldrich, St. Louis, MO, USA. SPSS version 11.5 software (IBM SPSS Statistics, Armonk, New York, USA) was used in statistical analyses and the results were presented in the form of means together with each of their standard deviations. Descriptive statistics were expressed as mean ± SD. The analysis of variance (ANOVA) was employed to examine the means of the experimental results, taking into consideration statistically significant differences at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

In this study, aqueous extracts were prepared with bee venom samples collected from different districts of Malatya (Table 1). The mean amounts of melittin, apamin, and phospholipase A2 contained in bee venom samples were found to be 46.38%±0.10%, 2.19±0.11%, and 12.46±0.19%, respectively (Table 2). It was determined that bee venom samples inhibited the collagenase enzyme by more than 50%.

Bee venom has many bioactive compounds in the chemical structure such as peptides (40 -50% melittin F and melittin derivatives, 2-3% Apamin, 1-2% Mast cell degranulation peptide - MCD, 0.5-2% secapin, 0.1% tertiapine, 1% adolapine, < 0.8% protease inhibitors, 1.4% procamine A & B, 13-15% minimin and cardiopeptin), enzymes (10-12% Phospholipase A2, 1-3% Hyaluronidase, 1% Acid phosphomonoesterase, 0.6% Alpha-Glucosidase, 1% Lysophospholipase), active amines (0.6-1.6% Histamine, 0.13-1% Dopamine, 0.1-0.7% Norepinephrine), sugars (2% Glucose and Fructose), lipids (4-5% 6-phospholipids), amino acids (1% A. amino acids, <0.5% Aminobutyric acid), minerals (3-4% P, Ca, Mg) and volatile compounds (4-8%) (Raghuraman and Chattopadhyay, 2007; Shimpi *et al*., 2016; Saleh, 2017; Altıntaş and Bektaş, 2019; Sig *et al*., 2019). Thanks to these compounds, bee venom has the potential of anti-inflammatory, anti-arthritis, antinociceptive, neuroprotective, antitumoral, antimicrobial, antidiabetic, and antirheumatic effects, both as a whole and separately with the purification of the components as mellitine (Altıntaş and Bektaş, 2019; Sig *et al*., 2019). Apamin phospholipase A2, and melittin, are major components of bee venom. The phospholipid packing is severely disordered when several melittin peptides roll up in the cell membrane, as a result of this cell lysis starts (Lee *et al*., 2015). Melittin activates both the lysis of nearly all plasmatic membranes and mitochondria intracellular membranes (Lee *et al*., 2017). Phospholipase A2 acts as melittin and both of them breaks up membranes of susceptible cells and improve their cytotoxic effect (Lee *et al*., 2015).

Apamin causes irreversibly blocking Ca^{2+}-activated K^+ (SK) channels and this is very important for pharmacological properties (Maylie *et al*., 2004). However, less known about the molecular mechanisms and the levels of gene regulation affected in the anti-inflammatory process of apamin (Lee *et al*., 2015). Konper *et al*. (2010) determined proteins in Carniol and Italian bee venoms using electrophoresis analysis in their study. It was stated that SDS-PAGE could be used for the determination and quantification of bee venom samples (Konper *et al*., 2010). Sirin *et al.* (2017) also investigated the usability of the SDS-PAGE electrophoresis technique in bee venom characterization. Findings of that research indicated that melittin amount of bee venom samples could be determined by using SDS-PAGE (Şirin *et al*., 2017). In the study conducted by Samanci and Kekeçoğlu (2019), melittin, phospholipase A2 and apamine contents of commercial and bee venom samples harvested directly from the hive were compared and the melittin, phospholipase A2 and apamine amounts of bee venom samples harvested directly from the hive were on average 40.91%, 10.78%, and 2.44%, respectively (Samancı and Kekeçoğlu, 2019). In another study, the apamin, phospholipase A2 and melittin contents of bee venom samples collected from Muğla province of Turkey were found as 4.05%, 14.36%, and 70.98%, respectively (Özgenç and Sevin, 2023). In a study conducted by Zidan *et al*. (2018), it was reported that the mellitin content of Egyptian bee venom was 46.71 and 67.44% using manually and electrical extracted methods while Carniolan bee venom was 33.71 and 56.54% using manual extracted method (Zidan *et al*., 2018). In another study, the main active ingredients identified in bee venom were apamin (2%) and melittin (48.7%) (Ahmed *et al*., 2024). In other study, the average amount of mellitin, phospholipase A2 and apamin were 40.25 ± 8.37%, 11.66 ± 2.77 and 2.31 ± 0.55%, respectively (Noori *et al*.,

2024). Although the usability of SDS-PAGE electrophoresis technique for the analyses of bee venom samples was investigated recently, it is not recommended method for this purpose. HPLC- UV/MS or LC-MS are methods used for quantification of bee venom contents.

In the literature, it was not reported before inhibition effect of bee venom on collagenase and myeloperoxidase *in vitro* but, the animal studies were performed generally (Hozzein *et al*., 2018; Namjou *et al*., 2022; Othman *et al*., 2023; Er-Rouassi *et al*. 2023). In a study, it was determined that bee venom activates antioxidant enzymes and supports diabetes wound healing (Hozzein *et al*., 2018). Namjou *et al*. (2022) determined the wound-healing properties of bee venom in diabetic rats in their study. According to this study, it was stated that bee venom has an important function in wound healing, but it can cause hepatotoxicity and nephrotoxicity (Namjou *et al*., 2022). Othman *et al*. (2023) determined the effect of bee venom on diabetic ulcers. They were reported that bee venom was effective on foot ulcers (Othman *et al*., 2023). Er-Rouassi *et al*. (2023) reported that bee venom and its major components affected facial nerve injury- induced in mice.

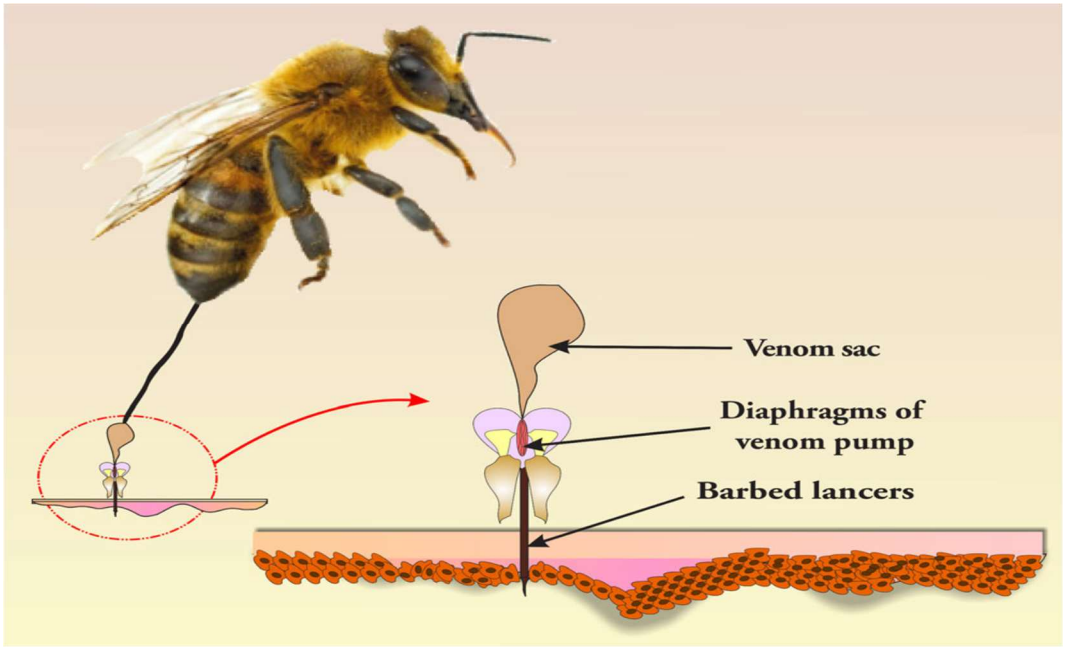

Figure 1. Honey bee poison and parts of sting (Komi *et al*., 2018)

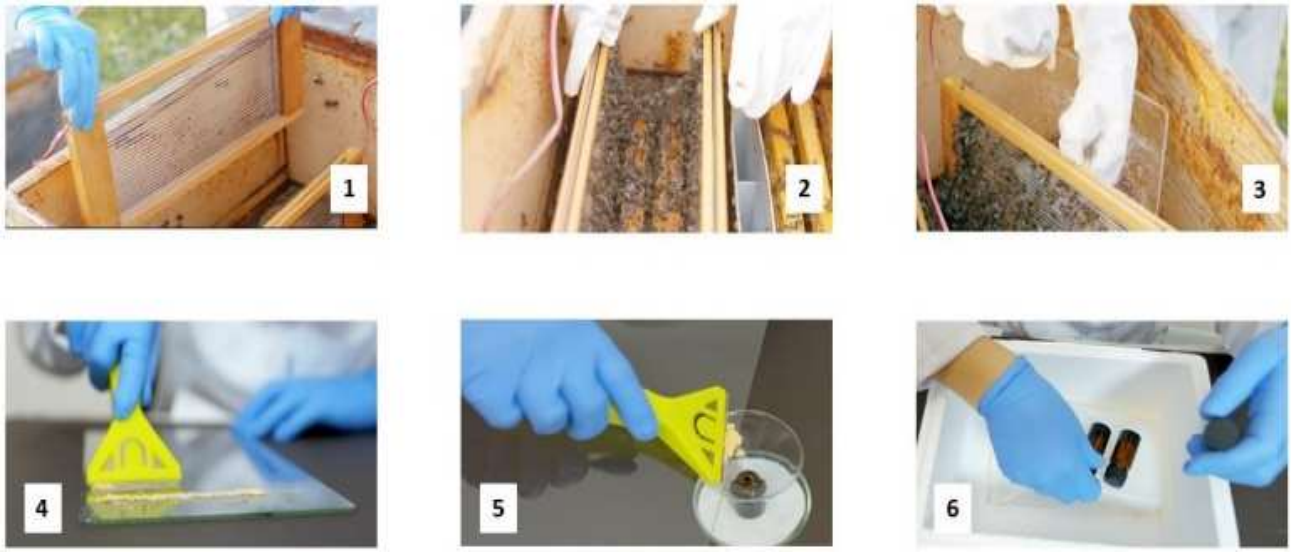

Figure 2. The scheme of bee venom harvesting 1 and 2 placing the collector, 3 collecting bee venom, 4-6 scraping and storing the bee venom (Çaprazlı and Kekeçoğlu, 2021).

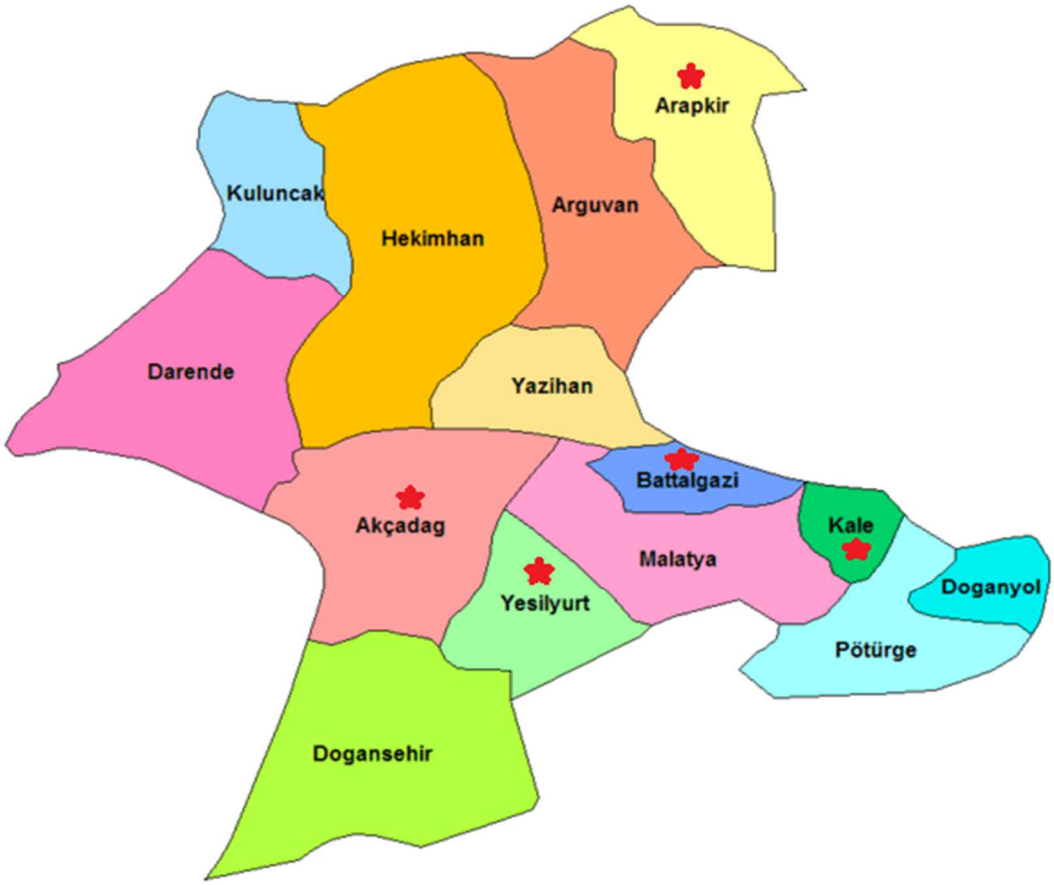

Figure 3. Malatya city of Turkey

Table 1. Coordinates of the district where the samples were collected.

Sample Number	District	Coordinates
1	Battalgazi	Latitude: 38.4234, Longitude: 38.3656 38° 25′ 24″ North, 38° 21′ 56″ East
2	Yeşilyurt	Latitude: 38.3, Longitude: 38.25 38° 18′ 0″ North, 38° 15′ 0″ East
3	Akçadağ	Latitude: 38.35, Longitude: 37.967 38° 21′ 0″ North, 37° 58′ 1″ East
4	Arapgir	Latitude: 39.033, Longitude: 38.483 39° 1′ 59″ North, 38° 28′ 59″ East
5	Kale	Latitude: 38.4161, Longitude: 38.7667 38° 24′ 58″ North, 38° 46′ 0″ East

Table 2. *Apis mellifera* L. bee venom protein content.

Sample Number	Total Protein Content (% per dry weight)	Melittin Content (%)	Phospholipase A2 Content (%)	Apamine Content (%)
1	67.27±1.19^{a}	45.18±0.09^{b}	11.63±0.18^{d}	1.98±0.07^{g}
2	70.18±1.23^{a}	46.27±0.09^{b}	12.60±0.2^{e}	2.08±0.09^{h}
3	73.67±1.08^{a}	47.58±0.10^{c}	13.75±0.18^{f}	2.30±0.09^{h}
4	72.44±1.28^{a}	47.09±0.12^{c}	12.44±0.18^{e}	2.19±0.09^{h}
5	68.13±1.27^{a}	45.78±0.12^{b}	11.86±0.20^{d}	2.42±0.10^{h}

*Different letters in the same columns show statistically differences between means ($p < 0.05$)

Table 3. Inhibition effects of *Apis mellifera* L. bee venom.

Sample Number	Collagenase Inhibition (%)	Myeloperoxidase Inhibition (%)
1	78.42±1.20[a]	28.96±0.98[d]
2	76.13±1.18[b]	25.71±0.95[e]
3	77.44±1.20[a]	28.75±0.97[d]
4	76.67±1.15[b]	28.69±1.08[d]
5	73.17±1.08[c]	26.44±0.087[e]

*Different letters in the same columns show statistically differences between means ($p < 0.05$)

Conclusion: Bee venom is an important natural product with a pharmaceutical effect, which is widely used in apitherapy applications with active ingredients. In this study, bee venom samples collected from different regions were characterized and their inhibition effects on collagenase and myeloperoxidase enzymes, which have an important role in wound healing, were determined for the first time. When the literature was examined, it was seen that there are not enough studies showing the effect of bee venom on the clinically important collagenase and myeloperoxidase enzymes *in vitro*. For this reason, the data obtained will contribute to both the literature and apitherapy practitioners. It will enable to use of bee venom directly in cosmetic products such as creams and tonics on skin regeneration and wound healing.

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