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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

INVESTIGATION OF THE ANTIMICROBIAL EFFECT OF HONEYBEE VENOMS (APITOXIN) FROM *APIS MELLIFERA CAUCASICA* AND *APIS MELLIFERA CARNICA*

Apis mellifera caucasica ve *Apis mellifera carnica* Irklarına Ait Arı Zehirlerinin (Apitoxin) Antimikrobiyal Etkisinin Araştırılması

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ABSTRACT

The discovery of new therapeutic agents is crucial in the fight against antimicrobial resistance. The antimicrobial potential of apitoxin from *Apis mellifera caucasica* and *A. m. carnica* (Hymenoptera: Apidae) was tested in vitro against Gram-positive (*Staphylococcus aureus* ATCC-25923, *Enterococcus faecalis* ATCC-29212), Gram-negative (*Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853) bacterial strains and a fungal pathogen (*Candida albicans* ATCC-10231). Using an electro stimulation technique, Apitoxin was extracted from honey bee colonies under standardized conditions between May 2022 and April 2023. The antimicrobial activity was evaluated using the disk diffusion method and the results were compared with standard antibiotics (ampicillin, vancomycin, trimethoprim-sulfamethoxazole, itraconazole) to calculate the antibiotic equivalence of the apitoxins. Apitoxin from both subspecies showed dose-dependent inhibitory effects against all microorganisms tested. The highest activity was observed against *E. coli*, with inhibition zone diameters of 16.6±0.2 mm for *A. m. caucasica* and 17.0±0.2 mm for *A. m. carnica* ($p<0.05$). No significant differences were found between subspecies in their effects on *E.coli*, *E.faecalis*, and *P.aeruginosa* ($p>0.05$). The results indicate that apitoxin has a broad spectrum of antimicrobial activity and could be used as a therapeutic agent.

Keywords: Apitoxin, Antimicrobial activity, *Apis mellifera caucasica*, *Apis mellifera carnica*

ÖZ

Antimikrobiyal dirençle mücadelede yeni terapötik ajanların keşfi önem taşımaktadır. Bu çalışmada, *Apis mellifera caucasica* ve *A. m. carnica* (Hymenoptera: Apidae) alt türlerinden elde edilen apitoksinin antimikrobiyal potansiyeli, Gram-pozitif (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), Gram-negatif (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) bakteri suşları ve bir fungal patojen (*Candida albicans* ATCC 10231) mikroorganizmalar üzerinde *in vitro* olarak değerlendirilmiştir. Mayıs 2022-Nisan 2023 tarihleri arasında standardize koşullarda yetiştirilen arı kolonilerinden elektrostimülasyon tekniğiyle apitoksin ekstrakte edilmiştir. Antimikrobiyal aktivite disk difüzyon yöntemiyle değerlendirilmiş ve sonuçlar standart antibiyotiklerle (ampisilin, vankomisin, trimetoprim-sülfametoksazol ve itrakonazol) karşılaştırılarak apitoksinlerin

antibiyotik eşleniği hesaplanmıştır. Her iki alt türden elde edilen apitoksin, test edilen tüm mikroorganizmalara karşı doza bağımlı inhibitör etki göstermiştir. En yüksek etki *E. coli*'ye karşı gözlemlenmiş olup, inhibisyon zon çapları *A. m. caucasica* için 16,6 ± 0,2 mm ve *A. m. carnica* için 17,0 ± 0,2 mm olarak ölçülmüştür (p<0.05). *E. coli*, *E. faecalis* ve *P. aeruginosa* üzerindeki etkilerde alt türler arasında anlamlı fark bulunmamıştır (p>0.05). Sonuçlar, apitoksinin geniş spektrumlu antimikrobiyal aktiviteye sahip potansiyel bir terapötik ajan olarak değerlendirilebileceğini göstermektedir.

Anahtar Kelimeler: Apitoksin, Antimikrobiyal aktivite, *Apis mellifera caucasica, Apis mellifera carnica*

GENİŞLETİLMİŞ ÖZET

Amaç: Bu araştırmanın amacı, *Apis mellifera caucasica* ve *A. m. carnica* alt türlerinden izole edilen apitoksinlerin antimikrobiyal potansiyelinin karşılaştırmalı olarak değerlendirmektir. Çalışma kapsamında apitoksinlerin Gram-pozitif ve Gram-negatif bakteriler ile fungal patojenlere karşı etkinliği incelenmiş, ayrıca standard antimikrobiyal ajanlarla karşılaştırmalı analizleri yapılarak antibiyotik eşdeğerlik değerleri belirlenmiştir.

Gereç ve Yöntem: Araştırma, Mayıs 2022-Nisan 2023 periyodunda standardize koşullarda yetiştirilen arı kolonileri üzerinde yürütülmüştür. Apitoksin ekstraksiyonu, modifiye elektrostimülasyon tekniği kullanılarak 15 dakikalık periyotlar halinde ve 1/15 günlük aralıklarla altı ardışık uygulama şeklinde gerçekleştirilmiştir. Elde edilen apitoksin örnekleri fizyolojik tuz çözeltisinde (0,9% NaCl) 8 mg/mL konsantrasyonunda süspanse edilmiş ve 0,22 µm por çaplı membran filtrasyonla sterilize edilmiştir. Antimikrobiyal aktivite testlerinde referans suşlar olarak *Staphylococcus aureus* ATCC-25923, *Enterococcus faecalis* ATCC-29212, *Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853 ve *Candida albicans* ATCC-10231 kullanılmıştır. Antimikrobiyal etkinlik, Kirby-Bauer disk difüzyon yöntemi ile değerlendirilmiş ve kontrol ajanları olarak standart antibiyotikler (ampisilin, vankomisin, trimetoprim-sülfametoksazol, itrakonazol) kullanılmıştır. Verilerin istatistiksel analizi tek yönlü varyans analizi (ANOVA) ve post hoc Tukey testi ile gerçekleştirilmiş, p<0.05 değeri istatistiksel anlamlılık sınırı olarak kabul edilmiştir.

Bulgular: Çalışmada test edilen her iki apitoksin preparatı, tüm mikroorganizmalara karşı doza bağımlı inhibitör etki göstermiştir. Maksimum antimikrobiyal aktivite *E. coli* üzerinde kaydedilmiş olup en yüksek konsantrasyonda inhibisyon zon çapları *A. m. carnica* için 17.0±0.2 mm ve *A. m. caucasica* için 16.6±0.2 mm olarak ölçülmüştür (p<0.05). Antimikrobiyal aktivitenin seyreltme oranı ile ters orantılı olduğu gözlenmiştir. Mikroorganizmaların apitoksine karşı duyarlılıkları değerlendirildiğinde, en yüksek duyarlılığın *E. coli*'de olduğu, bunu sırasıyla *E. faecalis*, *S. aureus* ve *C. albicans*'ın izlediği, en düşük duyarlılığın ise *P. aeruginosa*'da olduğu belirlenmiştir. Standart antibiyotiklerle karşılaştırmalı analizlerde, apitoksinlerin antibiyotik eşlenek değerleri şu şekilde saptanmıştır: *E. coli* için trimethoprim-sülfametoksazol eşleniği *A. m. carnica* ve *A. m. caucasica*'da sırasıyla 0,6 mg/mL ve 0,62 mg/mL; *S. aureus* için vankomisin eşleniği 0,72 mg/mL ve 0,84 mg/mL; *E. faecalis* için ampisilin eşleniği 0,36 mg/mL ve 0,38 mg/mL olarak belirlenmiştir. *P. aeruginosa*'ya karşı trimetoprim-sülfametoksazol eşleniği *A. m. carnica* ve *A. m. caucasica* için sırasıyla 0,48 mg/mL ve 0,52 mg/mL, ampisilin eşleniği ise 0,1 mg/mL ve 0,6 mg/mL olarak hesaplanmıştır. *C. albicans* için itrakonazol eşleniği *A. m. carnica*'da 0,3 mg/mL, *A. m. caucasica*'da 0,24 mg/mL olarak tespit edilmiştir.

Sonuç: Bu araştırma, farklı *A. mellifera* alt türleri arasında apitoksinlerin antimikrobiyal aktivitelerinin karşılaştırmalı analizini sunan ilk çalışmadır. Elde edilen veriler, apitoksinin geniş spektrumlu antimikrobiyal aktivite gösterdiğini ve terapötik ajan olarak potansiyel değer taşıdığını ortaya koymaktadır. Alt türler arasında spesifik patojenlere karşı etkinlik farklılıkları tespit edilmiştir. Antimikrobiyal aktivite analizlerinde *A. m. carnica*'dan elde edilen apitoksin *S. aureus*'a karşı sayısal olarak daha yüksek inhibisyon göstermiş ancak bu fark istatistiksel olarak anlamlı bulunmamıştır (p>0.05). *A. m. caucasica*'dan elde edilen apitoksin ise *C. albicans*'a karşı istatistiksel olarak anlamlı düzeyde daha güçlü antifungal etki sergilemiştir (p<0.05). Apitoksinin antimikrobiyal potansiyelinin tam olarak karakterize edilebilmesi için farklı coğrafi bölgelerden ve mevsimlerden elde edilen örneklerin değerlendirildiği, çevresel faktörlerin etkilerinin incelendiği ve *in vivo* etkinliğin araştırıldığı ileri çalışmalara ihtiyaç duyulmaktadır.

INTRODUCTION

Antimicrobial resistance (AMR) is seen as an increasing threat worldwide. Drug-resistant infections have become a critical challenge to human health due to the inadequacy of existing chemotherapeutic agents and the challenge of developing new antimicrobial agents. The World Health Organization has reported that the mortality rate associated with antimicrobial resistance is expected to be higher than cancer-related mortality by 2050 (O'Neill 2014, WHO 2022). This situation has necessitated the development of novel and effective antimicrobial agents. Endogenous antimicrobial peptides (AMPs) have been identified as bioactive molecules with high therapeutic potential as they are recognized as important components of the innate immune system of various organisms (Aşkar and Aşkar 2017). Apitoxin, which is extracted from *Apis mellifera*, has been identified as a collection of natural compounds with biological activity used for therapeutic purposes. (Tanuğur-Samancı and Kekeçoğlu 2021).

The antimicrobial, hepatoprotective, antioxidant, anti-inflammatory, antineoplastic, antiarthritic, radioprotective, cytoprotective and neuroprotective properties of apitoxin have been demonstrated *in vitro* and *in vivo* studies (Mizrahi and Lensky 1997, Münstedt and Bogdanov 2009). Apitoxin, which plays a crucial role in the defense mechanism of bee colonies and is synthesized in venom glands and stored in the venom sac, with one worker bee containing an average of 0.15-0.30 mg of venom (Crane 1990, Çaprazlı and Kekeçoğlu 2021, Schumacher *et al*. 1989). Apitoxin consists of various peptides, proteins, amino acids, enzymes, carbohydrates, essential oils and mineral components, with melittin (40-60% of dry weight) and phospholipase A2 (10-12% of dry weight) being the main components, along with apamin, histamine, dopamine and epinephrine. The chemical composition of apitoxin is known to vary depending on parameters such as bee subspecies, nutritional factors, ecological conditions and extraction methods (Karimi *et al*. 2012, Moreno and Giralt 2015, Wehbe *et al*. 2019).

Melittin is characterized as a peptide with bacterial cell membrane destabilizing properties and is the predominant active constituent in the apitoxin content. This peptide is characterized by its broad spectrum of antimicrobial, antifungal, antiviral, anticancer and neuromodulatory properties (Bogdanov 2015, Ownby *et al*. 1997, Park *et al*. 2010, Wang *et al*. 2009). The complex composition and versatile biological activity of apitoxin suggest a broad therapeutic potential. However, a gap was identified in the literature regarding the comparative analysis of the antimicrobial activities of apitoxins from different subspecies of *A. mellifera*. The aim of this study was to compare the antimicrobial activity of toxins (apitoxin) from *Apis mellifera caucasica* and *A. m. carnica* (Hymenoptera: Apidae) against various pathogenic gram-negative and gram-positive bacteria and fungi, including *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*, and to compare these effects with the antibiotics commonly used in the treatment of these pathogens (ampicillin, vancomycin, trimethoprim-sulfamethoxazole and itraconazole).

MATERIALS AND METHODS

This study was conducted in two phases between May 2022 and April 2023. In the first phase, bees of *A. m. caucasica* and *A. m. carnica* (Hymenoptera: Apidae) breeds were obtained from the Karabuk Province Beekeepers Association, which was authorized by the Ministry of Agriculture and Forestry. The specimens were housed in separate hives which were maintained throughout the summer season and their venom was collected. Venom extraction was carried out using a low-current electrostimulation method. In the second phase, the antimicrobial efficacy of the venoms against *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus* and *C. albicans* was investigated using the disk diffusion method. The colonies of *A. m. caucasica* and *A. m. carnica* were placed in standard Langstroth hives with southern exposure. The taxonomic analysis of the colonies was based on wing vein patterns and metric body measurements (Ruttner 1988).

Apitoxin extraction was carried out between June and August 2022 using a modified version of the electrostimulation method described by Benton *et al*. (1963). A weak electric current (3.0 mA, 1 Hz) from a 12 V DC source was applied to a fine wire mesh placed at 0.5 cm intervals on a glass plate (20x30 cm) and the device was positioned in the hive. Each extraction session lasted 15 minutes and was repeated six times at 1/15 day intervals. To minimize the risk of contamination, the glass plates were

covered with stretch film before extraction (Fakhim 1998).

The crystallized apitoxin on the glass plates was taken to the laboratory and collected mechanically with a spatula. The apitoxins from different bee breeds were separately placed in Eppendorf tubes and dissolved in 0.9% NaCl solution to achieve a final concentration of 8mg/mL. The solution was sterilized through a membrane filter with a pore diameter of 0.22 µm and stored under cryogenic conditions at -20 °C until use. Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and the fungus *Candida albicans* ATCC 60192 were used for the antimicrobial activity tests. The standard strains were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). The bacterial strains were cultured with Mueller-Hinton Broth and Mueller-Hinton Agar (Merck), while *C. albicans* was cultured with Sabouraud Dextrose Agar (Difco) and Sabouraud Dextrose Broth (Oxoid). The apitoxins of *A. m. caucasica* and *A. m. carnica* were weighed with analytical precision to 8mg/mL and placed in separate Eppendorf tubes. Each tube was filled with 1 mL of sterile physiological saline (0.9% NaCl) and homogenized by vortexing for 10-12 seconds. Serial dilutions of 10^{1} to 10^{10} were then prepared for each apitoxin preparation (Patel *et al*. 2015). The antimicrobial activity was evaluated using the Kirby-Bauer disk diffusion method (CLSI 2012). The microbial suspensions were adjusted to 0.5 McFarland standard (10^{8} CFU/mL). Bacterial and yeast suspensions (100 µL) were plated in Petri dishes on Mueller-Hinton agar (for bacteria) or Sabouraud dextrose agar (for *C. albicans*). Sterile paper disks (6 mm diameter) were placed on the agar surface and each apitoxin sample (1 mg/mL) was applied at 15 µL/disk. Standard antimicrobials (ampicillin, vancomycin, trimethoprim-sulfamethoxazole, itraconazole) were used as positive controls and 0.9% NaCl as negative control. Specifically, trimethoprim-sulfamethoxazole (SXT25) and ampicillin (AM10) were used for *P. aeruginosa*, vancomycin (VA30) for *S. aureus*, ampicillin (AM10) for *E. faecalis*, trimethoprim-sulfamethoxazole (SXT25) for *E. coli* and itraconazole (ITC10) antibiotic plates for C. albicans. The bacterial plates were incubated at 37 °C for 24 hours, while the fungal plates were incubated at 30°C for 48 hours. The inhibition zones formed after incubation were measured in millimeters using a digital calliper. All experiments were performed in three independent replicates and results were expressed as arithmetic mean. The calculation of the equivalence of the antimicrobial substances was based on the logarithmic relationship between the diameters of the inhibition zones and the concentrations. The following formula was used for this calculation: $E = (\log C_2 - \log C_1) / (R_2 - R_1)$. *E: Equivalence; C_1: Concentration of the antibiotic (µg); C_2: Concentration of the apitoxin (µg);; R_1: Diameter of the inhibition zone of the antibiotic (mm); R_2: Diameter of the inhibition zone of the apitoxin (mm).* The equivalent concentration between two antimicrobial substances was determined using the known concentration and the resulting zone of inhibition of the reference substance and the concentration and the resulting zone of inhibition of the test substance (Andrews 2001, Barry *et al*. 1976).

Statistical analysis was performed using IBM SPSS Statistics 20 software. One-way analysis of variance (ANOVA) and post hoc Tukey tests were performed to assess differences between groups. p-value <0.05 was considered statistically significant. Further statistical analysis was performed to evaluate the magnitude of differences between the two subspecies using effect size calculations (Cohen's d) with 95% confidence intervals (CI).

RESULTS

It was found that the apitoxins obtained from the subspecies *Apis mellifera caucasica* and *A. m. carnica* showed concentration-dependent inhibitory effects against all microorganisms tested (Table-1). Of the microorganisms tested, the highest antimicrobial activity was found against *Escherichia coli*, and this effect was statistically significant compared to the other microorganisms tested ($p<0.05$, one-way ANOVA).

When the apitoxin concentration was lowered from 10^{0} to 10^{10} in *E. coli*, the diameter of the inhibition zone decreased from 17.0 mm to 6.6 mm in *A. m. carnica* and from 16.6 mm to 6.6 mm in *A. m. caucasica*. This observation shows that the effect of apitoxin on *E. coli* is strongly concentration-dependent. At the highest concentration (10^{0}), *E. coli* showed the largest zone of inhibition (*A. m. carnica*: 17.0 mm, *A. m. caucasica*: 16.6 mm), while the smallest zone of inhibition was shown by *P. aeruginosa* (*A. m. carnica*: 8.8 mm, *A. m. caucasica*:

9.4 mm at 10º dilution, Cohen's d = 3.00, 95% CI [1.19, 4.81])).

These results show that *E. coli* is the most sensitive and *P. aeruginosa* the the lowest sensitive microorganism to apitoxin. In the ranking of the sensitivity of the tested microorganisms to apitoxin, *E. coli* was identified as the most sensitive microorganism, followed by *E. faecalis*, *S. aureus* and *C. albicans*, while *P. aeruginosa* was identified as the least sensitive microorganism among the tested microorganisms. Against *E. coli*, the apitoxins of *A. m. carnica* and *A. m. caucasica* produced inhibition zones of 17.0 ± 0.2 mm and 16.6 ± 0.2 mm, respectively (at 10º dilution; d = 2.00, 95% CI [0.48, 3.52]). Both apitoxin preparations showed similar efficacy against *E. coli*, and the difference between the apitoxins was found to be statistically non-significant (p>0.05, Student's t-test). *Staphylococcus aureus and Enterococcus faecalis* showed moderate susceptibility to both apitoxins. The apitoxin of *A. m. carnica* showed higher antimicrobial activity against *S. aureus* than the apitoxin of *A. m. caucasica* (12.4 ± 0.2 mm vs. 11.4 ± 0.2 mm at 10º dilution; Cohen's d = 5.00, 95% CI [2.48, 7.52]). However, this difference did not prove to be statistically significant (p>0.05, Student's t-test).

The apitoxin of *A. m. carnica* remained effective against E. *faecalis* even at a dilution of 10^9 (d = 1.00, 95% CI [-0.31, 2.31]), while its activity against *S. aureus* was limited to a dilution of 10^4. This indicates that it has higher efficacy compared to *E. faecalis*. The apitoxin from *A. m. caucasica* showed higher antifungal activity against *Candida albicans*. A more pronounced zone of inhibition was measured with the apitoxin from *A. m. caucasica* (12.0 ± 0.2 mm versus 10.4 ± 0.2 mm, at 10º dilution; (Cohen's d = 8.00, 95% CI [4.28, 11.72]), and this difference was found to be statistically significant (p<0.05, Student's t-test).

Table-1: Inhibition zones produced by the apitoxins of *A. m. carnica* and *A. m. caucasica* at different dilutions (mm)

	Dilutions	10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}
A. m. carnica	*E. coli*	17.0	13.4	10.8	10.2	9.2	7.8	7.4	7.4	6.6	-	-
	S. aureus	12.4	10.6	9.4	8.2	8.0	-	-	-	-	-	-
	E. faecalis	12.4	10.6	10.8	10.0	9.4	8.8	8.0	7.6	6.4	6.2	-
	P. aeruginosa	8.8	8.0	8.4	6.8	7.4	8.0	8.0	6.8	8.2	-	-
	C. albicans	10.4	9.4	7.8	6.8	-	-	-	-	-	-	-
A. m. caucasica	*E. coli*	16.6	12.8	11.0	10.2	9.6	9.0	8.4	8.2	7.6	7.0	6.6
	S. aureus	11.4	11.0	9.6	8.8	8.4	8.2	8.0	-	-	-	-
	E. faecalis	12.2	11.0	9.4	8.6	8.4	7.8	7.6	7.4	7.2	6.8	-
	P. aeruginosa	9.4	8.4	9.0	6.4	7.0	6.8	6.6	7.0	6.8	-	-
	C. albicans	12.0	8.8	7.8	7.2	6.8	-	-	-	-	-	-

8 mg venom dissolved in 1 ml of 0.9% NaCl; ± SD (SD = 0.2 mm for all measurements)

The antibiotics used to compare antimicrobial efficacy (ampicillin, vancomycin, trimethoprim-sulfamethoxazole and itraconazole) produced the expected zones of inhibition against all microorganisms tested. The zones of inhibition were measured as follows: Trimethoprim-sulfamethoxazole (SXT) and ampicillin (AM10), used for *P. aeruginosa,* produced zones of 15.6 mm and 8.2 mm, respectively; vancomycin (VA30), used for *S. aureus*, produced a zone of 20 mm; ampicillin (AM10), used for *E. faecalis* (AM10) produced a 28.8 mm zone; the sulfamethoxazole (SXT25) antibiotic disk used for *E. coli* produced a 37.6 mm zone; and the itraconazole (ITC10) disk used for *C. albicans* produced a 14 mm zone of inhibition.

As expected, no zones of inhibition were produced with the 0.9% NaCl solution used as a negative control (Table-2). Calculation of the equivalent concentrations of apitoxins to the antibiotics tested yielded the following results: against *P. aeruginosa*, *A. m. carnica* showed apitoxin at a concentration of 0.48 mg/mL and *A. m. caucasica* apitoxin at a concentration of 0.52 mg/mL for trimethoprim-sulfamethoxazole (SXT25) showed an equivalent effect, while for ampicillin (AM10) effective concentrations of 0.1 mg/mL for *A. m. carnica* apitoxin and 0.6 mg/mL for *A. m. caucasica* apitoxin were determined.

When the zones generated with standard antibiotic disks (SXT-AM) were compared with those of the

apitoxins, it was found that both apitoxins were more effective against *P. aeruginosa* than ampicillin ($p<0.01$). Equivalent activity against *E. coli* was shown by *A. m. carnica* apitoxin at 0.6 mg/mL and *A. m. caucasica* apitoxin at 0.62 mg/mL for sulfamethoxazole (SXT25). For vancomycin (VA30), which is used against *S. aureus,* effective concentrations of 0.72 mg/mL for *A. m. carnica* apitoxin and 0.84 mg/mL for *A. m. caucasica* apitoxin were determined. For ampicillin (AM10) used against *E. faecalis*, equivalent effects were observed at concentrations of 0.36 mg/mL for *A. m. carnica* apitoxin and 0.38 mg/mL for *A. m. caucasica* apitoxin.

Although higher apitoxin concentrations were required for efficacy against *E. coli*, *S. aureus* and *E. faecalis* compared to standard antibiotics, significant antimicrobial activity was still demonstrated ($p<0.01$). Against *Candida albicans*, *A. m. carnica* apitoxin at a concentration of 0.3 mg/mL and *A. m. caucasica* apitoxin at a concentration of 0.24 mg/mL showed comparable activity to itraconazole (ITC10) (Table-2).

Table-2. Inhibition zones produced by standard antibiotic disks and equivalent concentrations of *A. m. carnica* and *A. m. caucasica* apitoxins to the antibiotics tested

Microorganisms	Antibiotic	Inhibition Zone (mm)	Equivalent Concentration (mg/mL)	
			A. m. carnica	*A. m. caucasica*
E. coli	Trimethoprim-Sulfamethoxazole	37.6	0.6	0.62
S. aureus	Vancomycin	20.0	0.72	0.84
P. aeruginosa	Trimethoprim-Sulfamethoxazole	15.6	0.48	0.52
P. aeruginosa	Ampicillin	8.2	0.1	0.6
E. faecalis	Ampicillin	28.8	0.36	0.38
C. albicans	Itraconazole	14.0	0.3	0.24
Negative control	%0.9 NaCl	0	-	-

DISCUSSION

Infectious diseases are considered a major health problem, especially due to the emergence of drug resistance. Therefore, the development of effective new antimicrobial agents with novel mechanisms of action is considered essential. Bee venom (apitoxin) has been identified as an important defense mechanism of honeybees and is considered a promising natural agent for the treatment of cancer and other diseases due to its high biological activity potential. The antibacterial, antifungal and antiviral effects of bee venoms and their therapeutic potential have been demonstrated in numerous studies (El-Seedi *et al*. 2020, Hwang *et al*. 2022, Jadhav *et al.* 2024, Memariani and Memariani 2020).

The antimicrobial efficacy of bee venom is directly related to the composition of its bioactive components. Many studies in the literature have shown that major peptides, particularly melittin and phospholipase A_2, are responsible for this activity and that the differences in efficacy between subspecies are due to variations in the amount and ratios of these components. Previous studies investigating the composition of venoms of different Apis mellifera subspecies showed natural differences in the profiles of bioactive components between subspecies (El Mehdi *et al*. 2021, Małek *et al*. 2022). Therefore, these differences in composition underline the differences in antimicrobial efficacy we observed. While different antimicrobial activities have been reported for different Apis species such as *Apis cerana*, *A. dorsata* and *A. florea* (Surendra *et al.* 2011), there is insufficient data in the literature for a comparative analysis of the antimicrobial activities of apitoxins obtained from *Apis mellifera* subspecies. The antimicrobial activity of bee venoms from *Apis mellifera caucasica* and *Apis mellifera carnica* breeds was investigated against selected Gram-positive and Gram-negative pathogenic microorganisms, and differences in antimicrobial efficacy between the breeds were examined. According to our results, both apitoxin preparations showed concentration-dependent inhibitory effects against all microorganisms tested.

Tanuwidjaja *et al*. (2021) reported that the tested bee venom exhibited broad-spectrum antibacterial activity against all tested potentially pathogenic Gram-positive and Gram-negative bacteria. In

addition, Leandro *et al*. (2015) reported proportional inhibitory effects of apitoxin and its components against oral pathogens with increasing concentration. The concentration-dependent inhibitory effect of phospholipase A_2 contained in bee venom was documented by Boutrin *et al*. (2008).

These studies have shown that apitoxin has a broad spectrum and dose-dependent antimicrobial activity. The highest antimicrobial activity was observed against *Escherichia coli* ($p<0.05$). As reported by Isidorov *et al*. (2023), although bee venom shows high activity against both Gram-positive and Gram-negative bacteria, *E. coli* showed increased sensitivity to its antimicrobial effect. This increased sensitivity of *E. coli* to apitoxin is attributed to the membrane permeabilizing properties of its major components melittin, mast cell degranulation peptide (MCD) and phospholipase A_2, especially on the outer membrane of Gram-negative bacteria (Tanuwidjaja *et al*. 2021).

The apitoxins showed significant antimicrobial activity against *E. coli*, *S. aureus* and *E. faecalis* ($p<0.01$), albeit at higher concentrations than standard antibiotics. This result is consistent with other studies in literature. In addition, Boutrin *et al*. (2008) found that bee venom components, such as phospholipase A_2, showed significant antimicrobial activity against Gram-negative bacteria, especially *E. coli*, albeit at higher effective doses than antibiotics. Hegazi *et al*. (2017) reported strong antimicrobial effects of honey against *E. coli* and *S. aureus*, albeit at higher concentrations than standard antibiotics. These studies indicate the potential of apitoxin as an alternative agent against pathogenic microorganisms but also emphasize the need for further research on the optimal dosage and method of application. In our study, both apitoxins were found to be more effective against *P. aeruginosa* than ampicillin ($p<0.01$). Similarly, Dosler and Karaaslan (2014) reported synergistic antimicrobial effects against multidrug-resistant *P. aeruginosa* strains.

Al-Ani *et al*. (2018) reported that propolis extracts exhibited strong antimicrobial activity against *P. aeruginosa*, comparable to conventional antibiotics. These results suggest that apitoxin should be considered as an alternative or complementary agent for the treatment of *P. aeruginosa* infections, especially against resistant strains. The molecular mechanisms underlying the antimicrobial activity of bee venom primarily involve the disruption of membranes and the inhibition of cellular processes. The main component, melittin, interacts with bacterial cell membranes through its amphipathic structure and forms transmembrane pores that disrupt membrane potential and ion homeostasis, leading to cell death. Phospholipase A_2 catalyzes the hydrolysis of membrane phospholipids and acts synergistically with melittin to increase membrane permeability. In addition, melittin acts on intracellular targets by inhibiting DNA/RNA synthesis and ATP production, while other components such as apamin and MCD peptide contribute by modulating ion channels and inhibiting cell wall synthesis. These multiple mechanisms make it difficult for bacteria to develop resistance (Carpena *et al*. 2020, Stela *et al.* 2024).

While numerous studies have demonstrated the antimicrobial activity and therapeutic potential of bee venoms (El-Seedi *et al.* 2020, Hwang *et al.* 2022, Jadhav *et al*. 2024, Memariani and Memariani 2020), there is insufficient data in the literature for a comparative analysis of the antimicrobial activities of apitoxins extracted from *Apis mellifera* subspecies. Our study revealed certain differences in the antimicrobial activity of apitoxins from *A. m. caucasica* and *A. m. carnica* subspecies. The apitoxin from *A. m. carnica* showed higher antimicrobial activity against S. aureus, while the apitoxin from *A. m. caucasica* showed higher activity against *C. albicans* at the same dilution factor ($p<0.05$).

Our study underlines not only the potential use of apitoxin as an alternative agent against pathogenic microorganisms, but also the importance of selecting bee subspecies. Although the antimicrobial efficacy of various bee products, including honey, propolis and perga, against pathogenic microorganisms has been extensively studied, few studies have been conducted on the antimicrobial efficacy of bee venom. This study was the first to investigate the different antimicrobial efficacy of bee breeds with respect to their venom composition. The lack of LC-MS or HPLC analysis in our study can be considered a limitation. These analysis could have revealed detailed compositional differences between the subspecies. However, the role of bioactive components in antimicrobial efficacy, which is well described in the literature, and the natural variations between subspecies explain the biochemical basis of the differences in efficacy we observed.

Further studies with different bee breeds and microorganisms still need to be conducted. In addition, comparative analysis of the antimicrobial efficacy of bee venoms from different geographical regions and seasons should be conducted to understand the effects of environmental factors. Moreover, analysis of protein composition by LC-MS or SDS-PAGE would be crucial to identify possible variations in protein profiles between different apitoxin samples. Furthermore, the antimicrobial efficacy of bee venoms needs to be investigated in vivo models to contribute to the growing literature on alternative antimicrobial agents.

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Data Availability: The data used in this study can be obtained from the corresponding author upon reasonable request. Raw data from bee venom samples and antimicrobial test results are available in laboratory records.

Declaration: We declare that this manuscript has not been published elsewhere and has not been submitted for publication elsewhere. The authors are responsible for the accuracy of all information provided in this study.

Ethics Statement: This study does not require ethical approval as it only involves antimicrobial activity tests conducted under laboratory conditions.

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