http://iahsj.kaums.ac.ir/
International Archives of Health Sciences. 2024; 11(1): 36-41
https://doi.org/10.48307/IAHSJ.2023.413997.1053

Research Article Open Access

Evaluation of in-vitro antibacterial effect of crude venom of *Pseudocerastes Persicus* snake

Behrooz Fathi [1] *, **Abdoulah Jamshidi** [2], **Sadaf Sabzevari** [1]

[1] Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[2] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

* **Corresponding author:** Behrooz Fathi, Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. **Email:** behrooz048@gmail.com, b-fathi@um.ac.ir

Received: 5 September 2023 **Revised:** 19 December 2023 **Accepted:** 19 December 2023 **e-Published:** 1 February 2024

Abstract

Objectives: This study aimed to assess the antibacterial activity of the crude venom of *Pseudocerastes persicus* against some Gram-negative and Gram-positive bacteria using an antimicrobial susceptibility test.

Methods: The susceptibility of Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes*, *Bacillus subtilis*, *Salmonella typhimurium*, and *E. coli* O157:H7 to the crude venom of *Pseudocerastes persicus* was investigated at a concentration of 100 µg/ml. Standard antibiotic disks were utilized as positive controls. Furthermore, the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and MRSA was determined through the dilution method (160-1.25 µg/ml). These MIC values were compared with those of conventional drugs such as streptomycin (25 µg), tetracycline (30 µg), and neomycin (25 µg).

Results: The crude venom exhibited significant antibacterial activity against *Staphylococcus aureus*, MRSA, *Listeria monocytogenes*, and *E. coli* O157:H7. It displayed moderate effects on *Salmonella typhimurium* but showed no significant impact on *Bacillus subtilis*. The MIC values against these bacteria ranged from 160 to 80 µg/ml.

Conclusion: The venom from *Pseudocerastes persicus* demonstrates antibacterial properties and shows potential therapeutic value. Further investigations involving fractionation are necessary to fully explore its therapeutic potential.

Keywords: *Pseudocerastes Persicus*, Snake, Antibacterial effect.

Introduction

The rise of bacterial resistance to existing antibiotics is a pressing global crisis and a critical challenge in combating infectious diseases. Resistance is dynamic, evolving, and spreading, leading to diminished antimicrobial efficacy.[1-3] Bacterial infections now involve multidrug-resistant strains, underscoring the urgent need to explore and develop novel and effective antibacterial agents.[4,5]

Natural products serve as a rich source of medicinal compounds, with various organisms producing bioactive substances that have shown potential in combating bacteria.[6,7] Recently, compounds derived from venomous animals have emerged as promising therapeutic candidates, exhibiting toxicological, biochemical, physiological, and pharmacological properties that make them valuable for pharmaceutical research and drug discovery. These compounds comprise a complex mix of peptides and proteins with potent, selective, and specific activities against their molecular targets.[6-9]

Pharmacologically active compounds derived from venom have contributed to the development of FDA-approved drugs such as captopril, enalapril, ziconotide, batroxobin, eptifibatide, and tirofiban for various medical conditions.[10,11] Numerous studies have investigated the antibacterial properties of venom from diverse venomous creatures, including snakes,[7,12] scorpions,[13,14] honey bees,[14] wasps,[7,15] and spiders.[16,17]

Iran is home to 27 species of venomous snakes, some of which are medically significant and responsible for snakebite incidents, particularly those belonging to the viperidae family.[18] According to a retrospective study conducted by Dehghani et al., there were 67 reported cases of snakebite-related deaths over a ten-year period.[19] Among these venomous snakes is the Persian horned viper, scientifically known as *Pseudocerastes persicus* (DUMÉRIL, BIBRON & DUMÉRIL 1854). This highly

dangerous viper is found in the Middle East and Asia, including various provinces in Iran and neighboring countries.[18,20] The venom of *Pseudocerastes persicus* is classified as haematotoxic, impacting the circulatory system and its components. Despite its significance, only a few studies have explored the composition of *Pseudocerastes persicus* venom and its antibacterial properties.[20-22]

Bacterial infections present substantial health risks, including antibiotic resistance, hospital-acquired infections, and foodborne illnesses. Understanding and addressing these risks are vital to safeguarding human health. *Staphylococcus aureus*, commonly residing on the human body asymptomatically, can lead to infections, particularly in healthcare settings.

The use of methicillin in medical treatments has led to the emergence of a resistant bacterium known as Methicillin-Resistant *Staphylococcus aureus* (MRSA), significantly increasing morbidity and mortality rates. Since its discovery in 1961, MRSA has become a major global health concern due to its exceptional antibiotic resistance, resulting in severe systemic infections such as sepsis and pneumonia, posing a life-threatening risk.[23-25]

Listeria monocytogenes, a Gram-positive foodborne pathogen, can thrive inside cells and be transmitted through the consumption of dairy and poultry products. It is responsible for severe and life-threatening infections, including Listeriosis, leading to increased hospitalization and mortality rates.[26]

Bacillus subtilis is an important Gram-positive bacterium found in soil, water, and the gastrointestinal tracts of humans and animals. It forms spores that enable survival in harsh conditions. This bacterium is well-studied and widely used in industrial applications, agriculture, and biotechnology.[27]

Salmonella typhimurium is a Gram-negative harmful bacterium that causes a serious illness called salmonellosis. It is easily spread through contaminated food or water and contact with infected animals or their feces. This bacterium is responsible for 20% of all food and waterborne illnesses in humans and often leads to acute gastroenteritis with watery diarrhea.[28]

E. coli, a Gram-negative bacterium, typically resides in the gastrointestinal tract as part of the normal flora. While some strains can cause illnesses, most are harmless or even beneficial. It is used as an indicator for evaluating water quality and contamination. *E. coli* is also valuable in biological research, probiotics, nutrient recycling, and decomposition.[29,30]

Objectives

The objective of this study was to investigate the impact of crude venom from *Pseudocerastes persicus* on the growth of various Gram-negative and Gram-positive bacteria, including *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus*, MRSA, *Bacillus subtillis*, and *Listeria monocytogenes*.

Methods

An antimicrobial susceptibility test was conducted using the disc diffusion and minimum inhibitory concentration (MIC) methods for this investigation. In the disc-diffusion method, bacterial cultures were allowed to grow overnight at 37°C in 20 ml of Muller Hinton (MH) broth (pH 7.4). A lyophilized venom of 100 µg was dissolved in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) and stored at 4°C for the assay. Susceptibility tests were performed by spreading 200 µl of bacterial culture containing 1.5×106 colony-forming units (CFU) ml-1 on sterile MH agar plates (90 mm diameter) using a sterile cotton swab. The surface of the medium was allowed to dry for approximately 3 minutes at room temperature. Sterile blank paper discs with a diameter of 7 mm were then placed on the MH agar surface, and 20 µl of the venom sample at the mentioned concentration was added per disc in five replicates. Three standard antibiotic discs containing streptomycin (25 µg), tetracycline (30 µg), and neomycin (25 µg) were used as positive drug controls. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured. The experiments were repeated with the venom five times. The testing was performed according to the guidelines set by the Clinical and Laboratory Standards Institute (CLSI).

The MIC was determined using the broth dilution method described by Wu and Hancock in 1999.[32] The bacteria were cultured in Muller Hinton Broth (MHB) for 24 hours until reaching a mid-logarithmic phase with an absorbance at A600 of 0.1-1.0 (equivalent to 1.5×10^6 to 3.2×10^8 CFU ml^{-1}). The venom was prepared at the required test concentrations of 160, 80, 40, 20, 10, 5, 2.5, and 1.25 µg/ml using Tris-HCl buffer (pH 7.4). In a 96-well plate, 20 µl of venom samples were added to 200 µl of the mid-logarithmic phase culture bacteria. Five independent experiments were performed as replicates.

One well containing 200 µl of an inoculated bacterial culture served as the bacterial control. Another well containing 200 µl of un-inoculated MH broth and 20 µl of Tris-HCl buffer (pH 7.4) was used as the negative control. To compare the antibacterial effect of the venom with a standard antibiotic, 20 µl of antibiotic solution in Tris-HCl buffer, along with the same volume of venom at

concentrations ranging from 1.25 to 160 µg/ml, were added to 200 µl of MH broth. The cultured plates were then incubated at 37°C for 24 hours. The inhibition of bacterial growth was determined using an ELISA reader (Molecular Devices Emax precision microplate reader; Research Instruments, Singapore) by measuring the absorbance at 560 nm.

The materials used for this study included the lyophilized crude venom of *Pseudocerastes persicus*, generously provided by the Razi Vaccine and Serum Research Institute in Tehran, Iran. Microorganisms included the MRSA strain obtained from Razi Culture Collection and the remaining mentioned strains from Mast Laboratories LTD-UK. Standard antibiotics tetracycline, neomycin, and streptomycin were obtained from Liofilchem S.R.L Diagnostic Company (Italy) and used for comparison with the venom.

Statistical Analysis

The data were analyzed using SPSS (version 16.0, SPSS Inc, Chicago, IL, USA) and Prism software. Friedman's non-parametric test was used to compare the effects of *Pseudocerastus p.* snake venom and antibiotics, with Dunn's post hoc test used to analyze the combined effects. The data were expressed as mean±standard deviation. A "P-value" less than 0.05 was considered significant.

Ethical considerations

The study was approved by the Committee of Ethics in Research at the Faculty of Veterinary Medicine and is registered with an assigned ID number (ID: 61078). The study was conducted in accordance with the Declaration of Helsinki.

Results

Effect on *Staphylococcus aureus*

There was no statistically significant variation in the impact between *Pseudocerastus* snake venom and streptomycin on *S. aureus* bacteria. However, a significant difference was observed with neomycin (P=0.037) and tetracycline (P=0.006), suggesting lower efficacy of the venom compared to these antibiotics on S. aureus bacteria [Table 1, Figure 1].

Effect on Methicillin-Resistant *Staphylococcus Aureus* (MRSA)

Similarly, there was no statistically significant variation in the effect between *Pseudocerastus* snake venom and streptomycin on MRSA bacteria, with significant differences for neomycin (P=0.005) and tetracycline (P=0.002) indicating lower efficacy of the venom compared to these antibiotics [Table 1, Figure 1].

Effect on *Listeria monocytogenes*

For *L. monocytogenes* bacteria, no statistically significant variation was observed between *Pseudocerastus* snake venom and streptomycin, while differences were significant for neomycin (P=0.003) and tetracycline (P=0.000), suggesting lower efficacy of the venom compared to these antibiotics [Table 1, Figure 1].

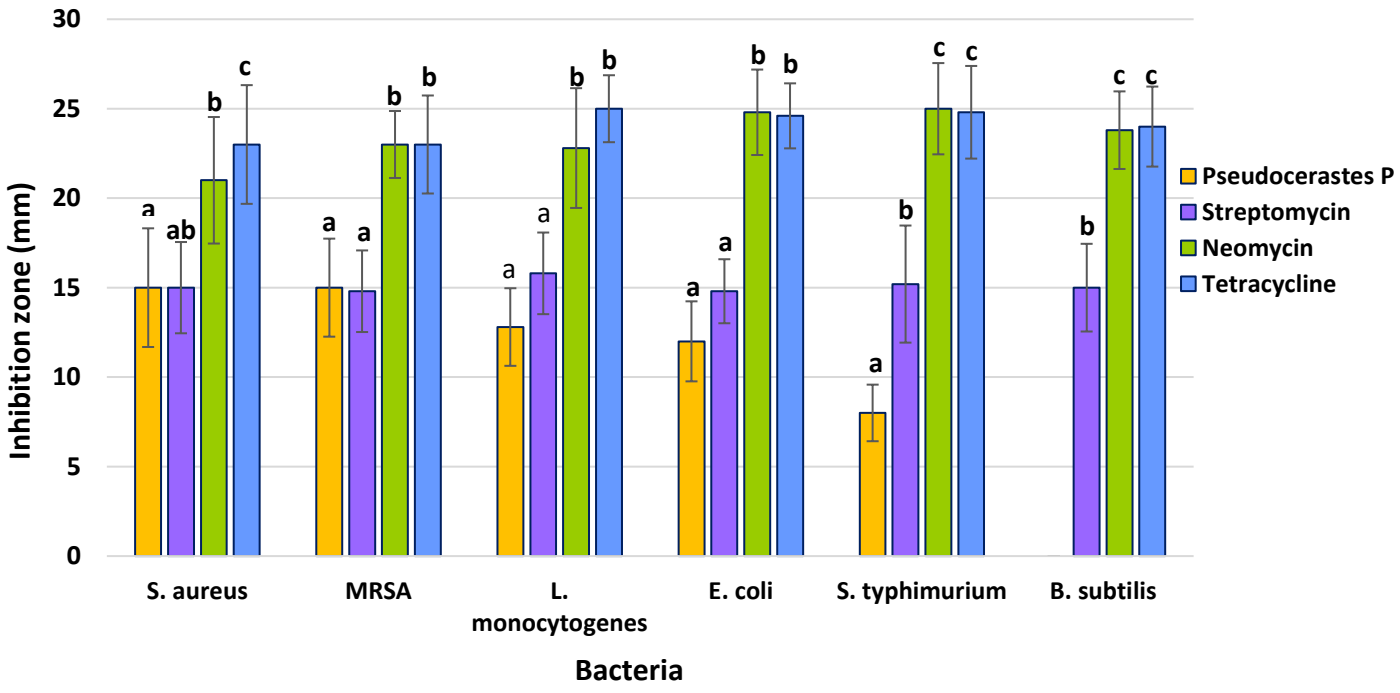

Figure 1. *In vitro* antibacterial activity of *Pseudocerastes Persicus* crude venom tested by disc-diffusion and compared to some standard antibiotics. Each number is presented as mean ± SD of the inhibition zone in mm (n =5).

Table 1. Antibacterial effect of *Pseudocerastes persicus* venom in comparison with tetracycline, neomycin and streptomycin on *Staphylococcus aureus*, *Methicillin-Resistance Staphylococcus Aureus* (MRSA), *Listeria monocytogenes*, *E. coli* O157.H7, *Salmonella typhimurium*, and *Bacillus subtilis*

Microorganism Antibiotics/Venom	*S. aureus*	*MRSA*	*L. monocytogenes*	*E. coli*	*S. typhimurium*	*B. subtilis*
Pseudocerastes P	15±3.32	15±2.74	12.8±2.17	12±2.24	8±1.58	0±0
Streptomycin	15±2.55	14.8±2.28	15.8±2.28	14.8±1.79	15.2±3.27	15±2.45
Neomycin	21±3.54	23±1.87	22.8±3.35	24.8±2.39	25±2.55	23.8±2.17
Tetracycline	23±3.32	23±2.74	25±1.87	24.6±1.82	24.8±2.59	24±2.24

Effect on *E. coli*

There was no statistically significant variation in the impact between *Pseudocerastus* snake venom and streptomycin on *E. coli* bacteria, with significant differences observed for neomycin (P=0.001) and tetracycline (P=0.002), indicating lower efficacy of the venom compared to these antibiotics.

Effect on *S. Typhimurium*

Significant variation was found in the impact between *Pseudocerastus* snake venom and all three antibiotics (streptomycin, neomycin, tetracycline) on *S. typhimurium* bacteria, suggesting lower efficacy of the venom compared to these antibiotics.

Effect on *B. subtilis*

Similarly, significant variation was observed in the impact between *Pseudocerastus* snake venom and all three antibiotics (streptomycin, neomycin, tetracycline) on *B. subtilis* bacteria, indicating a lack of efficacy of the venom compared to these antibiotics.

The results of minimum inhibitory concentration (MIC)

The MIC was determined for bacteria that exhibited higher sensitivity in the disc-diffusion test, including *Staphylococcus aureus* and MRSA. The MIC test was conducted using the dilution method with concentrations ranging from 1.25 to 160 µg/ml, and the observed effect was recorded at concentrations ranging from 80 to 160 µg/ml. The MIC values were compared to those of standard antibiotics, namely streptomycin, tetracycline, and neomycin.

Discussion

Venoms derived from venomous animals, such as snakes, have been reported to possess antibacterial properties.[4,5,7,33] The objective of this study was to assess the antibacterial activity of the crude venom extracted from the viper snake *Pseudocerastes persicus* against various gram-positive and gram-negative bacteria in vitro. The results demonstrated a significant antibacterial effect of the *Pseudocerastes persicus* venom against *Staphylococcus aureus*, MRSA, *Listeria monocytogenes*, and *E. coli* O157.H7 when compared to the standard antibiotic streptomycin. Furthermore, when compared to neomycin and tetracycline, the venom exhibited a significant effect specifically against *Staphylococcus aureus* and MRSA. It also showed a moderate effect against *E. coli* and *Salmonella typhimurium* but did not exhibit any antibacterial activity against *Bacillus subitilis* [Table 1 and Figures 1].

The MIC test, conducted using the dilution method (1.25-160 µg/ml), indicated the antibacterial effect of the venom at concentrations ranging from 80 to 160 µg/ml. A study by Jami et al. (2010) on the antibacterial activity of crude venom from the Iranian viper snake *Echis carinatus* also reported a significant effect against *Staphylococcus aureus* and MRSA with an MIC of 80 µg/ml for *Echis carinatus* against both strains, which aligns with our findings. However, their venom did not demonstrate any impact on other tested bacteria, including *Listeria monocytogenes*, *Bacillus subtilis*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7, which differs from our results.[33]

In another study conducted by Abtahi et al., the antibacterial effects of several snake venoms were evaluated against various pathogenic bacterial strains. A notable finding from this study was that two species of Viperidae (*P. persicus* and *P. urarachnoides*) and one species of Elapidae (*N. oxiana*) exhibited moderate antibacterial effects against *S. aureus*,[6] aligning with the observations recorded in the present study. Additionally, in 2007, Samy et al., reported that the venoms of four species of Viperidae (*D. russelli russelli*, *E. carinatus*, *B. gabonica rhinoceros*, and *B. arietans*) and two species of Elapidae (*P. australis* and *N. naja*) demonstrated effectiveness against both gram-positive and gram-negative bacteria, with the most potent antibacterial activity observed against *S. aureus*.[12] These findings regarding the antibacterial effects of viper venom are consistent with the results obtained in the current study.

In 2021, Nodooshan et al., conducted two separate

surveys on the crude venom and fractions of *Pseudocerastes persicus* snake venom. Their findings revealed that the crude venom exhibited the highest inhibitory effect against two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, at concentrations ranging from 12.5 to 400 µg/ml and 25 to 400 µg/ml, respectively. However, it only demonstrated an inhibitory effect against the gram-negative bacterium *Escherichia coli* at the highest concentration tested (400 µg). Additionally, two fractions of the snake venom displayed significant antibacterial activity against gram-positive bacteria compared to gram-negative bacteria.[21]

In 2020, Talebi conducted a study on the antibacterial effect of two isolated proteins from *Naja naja oxiana* venom, reporting significant antibacterial activity against two Gram-positive bacteria, *B. anthracis*, and *S. pneumonia*.[34] These variations in antibacterial activity may be attributed to various factors, including differences between bacterial species and the diverse structure of their cell walls.[21] According to the literature, molecular compounds such as proteins and enzymes found in venoms are responsible for various biological activities. Consequently, these substances may selectively target specific molecules in certain bacterial strains while not affecting others.[8, 21, 22]

Although the present study focused on the antibacterial effects of the crude venom of *Pseudocerastes persicus*, referring to the literature suggests that isolating fractions with varying molecular sizes and biochemical properties can enhance the investigation and increase the likelihood of discovering antibacterial components.

Conclusions

The venom of the *Pseudocerastes persicus* snake exhibits a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria. The results can be further improved by isolating, identifying, and characterizing the active antibacterial agents present in this venom. These agents can then be tested on other medically significant bacteria that have shown resistance to existing antibiotics, using different concentrations to obtain comprehensive findings.

Acknowledgment

We thank Razi Vaccine and Serum Research Institute, Tehran-Iran, for generously providing the Lyophilized crude venom of *Pseudocerastes persicus*.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

Methicillin-Resistant *Staphylococcus aureus*: MRSA;
Minimum inhibitory concentration: MIC;
Colony-forming units: CFU;
Clinical and Laboratory Standards Institute: CLSI;
Muller Hinton Broth: MHB.

Authors' contributions

FB and JA: Methodology, writing original draft, and supervised the research. SS: Performed the experimental process. All authors read and approved the final manuscript. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

Funding

This research was supported by funds from Ferdowsi University of Mashhad, Mashhad-Iran.

Role of the funding source

None.

Availability of data and materials

The data used in this study are available from the corresponding author on request.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Committee of Ethics in Research at the Faculty of Veterinary Medicine and is registered with an assigned ID number (ID: 61078).

Consent for publication

By submitting this document, the authors declare their consent for the final accepted version of the manuscript to be considered for publication.

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How to Cite this Article:
Fathi B, Jamshidi A, Sabzevari S. Evaluation of in-vitro Antibacterial Effect of Crude Venom of Pseudocerastes Persicus Snake. Int Arch Health Sci. 2024;11(1):36-41. doi: 10.48307/IAHSI.2023.413997.1053