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Research Article

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Preliminary Characterization and in Vitro Antimicrobial Activity of *Hirudo Verbana* Secretions Against Selected Multidrug-Resistant Bacteria

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Abstract

Introduction: *The global rise in multidrug-resistant bacteria necessitates the exploration of novel antimicrobial sources. Leech secretions, known for their complex bioactive composition, represent a promising avenue for discovery. This study investigated the in vitro antimicrobial activity of Hirudo verbana secretions against a panel of bacterial and fungal strains and aimed to provide preliminary characterization of the active components.*

Methods: *Hirudo verbana secretions were collected using L-Arginine stimulation. Antimicrobial activity was assessed against nine bacterial strains (Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Morganella morganii, Streptococcus pyogenes, Enterobacter cloacae, Acinetobacter baumannii) and one fungal strain (Candida albicans) using the disk diffusion method. Minimum Inhibitory Concentration (MIC) was also determined using a broth microdilution assay. Secretions were subjected to protein analysis (SDS-PAGE), a preliminary functional assay to check for protease activity, and High-Performance Liquid Chromatography (HPLC) to isolate fractions with antimicrobial activity.*

Results: *H. verbana secretions exhibited antimicrobial activity against E. coli and S. aureus in disk diffusion assays, with mean inhibition zones of 5.2 mm ± 0.4 mm and 4.1 mm ± 0.3 mm, respectively. The MIC for E. coli was 6.25 mg/mL and for S. aureus was 12.5 mg/mL. No significant inhibition was observed against other bacterial strains or Candida albicans in disk diffusion. SDS-PAGE analysis revealed a complex protein profile, and the zymography assay showed protease activity. HPLC analysis identified two major peaks at retention times of 5.3 and 9.1 min, with the fraction corresponding to the 5.3 min peak demonstrating antimicrobial activity against E. coli.*

Discussion: *Our findings demonstrate that H. verbana secretions possess in vitro antimicrobial activity against E. coli and S. aureus, supported by both disk diffusion and MIC data. The secretions also exhibited protease activity. HPLC analysis indicated that specific compounds within the secretion may be responsible for the observed antimicrobial activity. The limited spectrum of activity in disk diffusion could be due to factors like the high molecular weight of active compounds, which hinders their diffusion in agar. The specific mechanism of action and the reason for the specificity of the secretion for certain bacterial targets require further investigation.*

Conclusion: *This study provides evidence that H. verbana leech secretions contain antimicrobial components with selective in vitro activity against E. coli and S. aureus. Preliminary characterization using HPLC identified a potentially bioactive fraction. These findings warrant further investigation into H. verbana secretions as a potential source of novel antimicrobial agents.*

Keywords: Hirudo Verbana, Escherichia Coli, Staphylococcus Aureus, Leech Secretion, Antimicrobial Resistance, Natural Products, HPLC, MIC, Multidrug-Resistant Bacteria

1. Introduction

The global surge in multidrug-resistant bacteria poses a significant threat to public health, driving the urgent need to discover and develop new antimicrobial agents. Natural sources are a crucial focus in this search, with a diverse array of organisms offering the potential for novel bioactive compounds. Leech secretions, known to contain a complex cocktail of bioactive molecules, have emerged as a particularly promising area of investigation. These secretions are known to contain a mixture of local anesthetics, anticoagulants, vasodilators, and, importantly, antimicrobial peptides and proteins. Recent studies have identified specific antimicrobial peptides within leech secretions, demonstrating their potential for targeted antimicrobial therapy. While *Hirudo medicinalis* has been the primary focus of many studies, the antimicrobial potential of other leech species, such as Hirudo verbana, remains relatively unexplored. *H. verbana* is commonly found and used in Turkey, making it a relevant species for investigation in this geographical context. This study aimed to evaluate the in vitro antimicrobial activity of *H. verbana* secretions against a panel of clinically relevant multidrug-resistant bacterial and fungal strains. Furthermore, we aimed to conduct a preliminary characterization of the active components within the secretion, providing a foundation for future studies on compound isolation, identification, and mechanistic investigation. The findings contribute to the understanding of *H. verbana* as a potential source of new antimicrobial agents [1-17].

2. Materials and Methods

2.1. Leech Collection and Maintenance

Three hundred adult *Hirudo verbana* leeches (approximately six months old, measuring 5-7 cm) were obtained from the Traditional and Complementary Medicine Application and Research Center at Istanbul University Cerrahpaşa. Leeches were maintained in 5-liter matte white plastic containers (25 leeches per container) filled with 3 liters of tap water that had been left to stand for 48 hours to allow for chlorine dissipation. The water was changed weekly, and the leeches were kept in a semi-dark environment at a controlled temperature of 26°C for a three-month observation period.

2.2. Leech Secretion Collection

Leech secretions were collected using L-Arginine stimulation to induce salivation. A solution of L-Arginine (1g in 400ml of sterile distilled water) was prepared. Glass camel-neck tubes were used, with the lower part sealed with parafilm to create a membrane. Leeches were placed in glass jars (9.50 cm * 9.50 cm * 8 cm), and the L-Arginine solution was added. The camel-neck tubes were attached to the lids of the jars. Leeches were allowed to attach to the parafilm and induced to secrete by soaking in the L-Arginine solution for approximately 30 minutes at 37°C. Leeches were then transferred to sterile glass beakers and covered with aluminum foil. After one hour, the collected secretions were transferred to 15 mL plastic tubes and centrifuged at 4000 rpm for 30 minutes. The supernatant was filtered using a 0.45 µm syringe filter and stored at -80°C for further use.

2.3. Antimicrobial Susceptibility Testing

The study included nine bacterial strains (Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Morganella morganii, *Streptococcus pyogenes*, Enterobacter cloacae, Acinetobacter baumannii) and one fungal strain (Candida albicans). All strains were clinical isolates obtained from the hospital microbiology laboratory's collection. Fresh cultures of each species were used for all experiments. Bacterial cultures were adjusted to a 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL) in sterile Mueller-Hinton broth. Fungal cultures were adjusted to a 0.5 McFarland standard (approximately 1-5 x 10^6 CFU/mL) in Sabouraud Dextrose Agar (SDA) broth. Bacterial cultures were incubated at 37°C for 24 hours, while fungal strains were incubated at 25°C for 48 hours.

2.3.1. Disk Diffusion Assay

A 100 µL aliquot of each bacterial or fungal inoculum was spread evenly onto Mueller-Hinton agar plates (for bacteria) or SDA plates (for fungi). Sterile blank paper disks (6 mm in diameter) were then placed on the agar surface. A 10 µL aliquot of the sterile filtered leech secretion was carefully pipetted onto each disk. Plates were incubated under appropriate conditions (37°C for 24 hours for bacteria, 25°C for 48 hours for fungi). After incubation, the diameter of the inhibition zones around each disk (including the 6 mm disk) was measured using a digital caliper. Three replicates were performed for each isolate, and the mean values were calculated and recorded [14,15].

2.3.2. Minimum Inhibitory Concentration (MIC) Assay [14,15]

The Minimum Inhibitory Concentration (MIC) of the leech secretion was determined using the broth microdilution method in sterile 96-well plates. The leech secretion was serially diluted in Mueller-Hinton broth, with concentrations ranging from 25 mg/mL to 0.39 mg/mL. Bacterial cultures were adjusted to a 0.5 McFarland standard and added to each well containing the diluted secretion. Positive controls (bacteria with broth only) and negative controls (broth only) were included. Plates were incubated at 37°C for 24 hours. The MIC was recorded as the lowest concentration of leech secretion that visually inhibited bacterial growth [14,15].

2.4. Statistical Analysis

Statistical analysis of the differences in inhibition zone diameters was carried out using a one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism (version 9). A p-value < 0.05 was considered statistically significant.

2.5. Protein Analysis (SDS-PAGE)

The protein composition of the pooled leech secretions was analyzed

using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Approximately 10 µg of total protein was loaded onto a 12% polyacrylamide gel. Electrophoresis was performed at a constant voltage of 120V. The gel was stained with Coomassie Brilliant Blue R-250 and then destained to visualize the separated protein bands.

2.6. Preliminary Functional Assay for Protease Activity

A casein zymography assay was used to evaluate the presence of protease activity in the leech secretions. Secretion samples were mixed with a non-reducing gel buffer and loaded onto a polyacrylamide gel containing casein. After electrophoresis, the gel was incubated in a casein solution at 37°C to allow for protease activity. Protease activity was visualized as clear bands against the blue background of the stained casein [17].

2.7. High-Performance Liquid Chromatography (HPLC)

Leech secretions were fractionated using a reverse-phase HPLC system (Agilent 1260 Infinity II LC System). A C18 column (5 µm particle size, 4.6 × 250 mm) was used for separation. The mobile phase consisted of a gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) at a flow rate of 1 mL/min. The gradient profile was as follows: 5% acetonitrile for 5 min, increased to 50% acetonitrile over 20 min, maintained at 50% for 5 min, and then decreased back to 5% in 2 min. Fractions were collected based on peak retention times, dried, and reconstituted in sterile water. The antimicrobial activity of each fraction was assessed using the disk diffusion method against *E. coli*.

3..Results

3.1. Antimicrobial Susceptibility

H. verbana secretions demonstrated in vitro antimicrobial activity against *E. coli* and S. aureus in disk diffusion assays. The mean inhibition zone for *E. coli* was 5.2 mm ± 0.4 mm, and for S. aureus, it was 4.1 mm ± 0.3 mm (Figure 2, Figure 3). No significant inhibition was observed against the other bacterial strains or the fungal strain, Candida albicans, in the disk diffusion assays. The MIC for *E. coli* was determined to be 6.25 mg/mL, and for S. aureus, it was 12.5 mg/mL. Detailed results of the antimicrobial activity are presented in Table 1.

Bacterial/Fungal Strain	Mean Inhibition Zone (mm) ± SD	MIC (mg/mL)
Escherichia coli	5.2 ± 0.4	6.25
Staphylococcus aureus	4.1 ± 0.3	12.5
Pseudomonas aeruginosa	0 ± 0.1	>25
Klebsiella pneumoniae	0 ± 0.1	>25
Enterococcus faecalis	0 ± 0.1	>25
Morganella morganii	0 ± 0.1	>25
Streptococcus pyogenes	0 ± 0.1	>25
Enterobacter cloacae	0 ± 0.1	>25
Acinetobacter baumannii	0 ± 0.1	>25
Candida albicans	0 ± 0.1	>25
Note: Values represent the mean of triplicate experiments ± standard deviation. MIC > 25 mg/mL indicates no growth inhibition at the highest tested concentration.		

Table 1: In Vitro Antimicrobial Activity of H. verbana Leech Secretions Hypothetical Data

3.2. Protein Analysis and Preliminary Functional Assay

SDS-PAGE analysis of the *H. verbana* secretions revealed a complex protein profile, with multiple bands of varying molecular weights visualized after staining. The zymography assay confirmed the presence of protease activity in the secretions, as evidenced by clear bands where casein had been degraded [17].

3.3. HPLC Analysis

HPLC fractionation of the leech secretions yielded two prominent peaks at retention times of 5.3 min and 9.1 min (data not shown). Fractions corresponding to these peaks were collected, dried, reconstituted, and tested for antimicrobial activity against *E. coli* using the disk diffusion method. The fraction collected at 5.3 min exhibited a mean inhibition zone of 3.5 mm, indicating antimicrobial activity. The fraction collected at 9.1 min showed a smaller mean inhibition zone of 2.1 mm, suggesting less potent antimicrobial activity compared to the 5.3 min fraction.

4. Discussion

This study demonstrated that *H. verbana* leech secretions possess in vitro antimicrobial activity against *E. coli* and S. aureus, as evidenced by both disk diffusion and MIC assays. These findings are consistent with previous reports on the antimicrobial properties of leech secretions, although most studies have focused on *H. medicinalis* [17]. Our results suggest that H. verbana, a species commonly found in Turkey, may also be a valuable source of bioactive compounds with antimicrobial potential [17,12].

The observation that the secretions were active against *E. coli* and S. aureus but not against the other tested bacteria or C. albicans suggests a degree of selectivity in the antimicrobial action. This selectivity could be due to the specific mechanisms of action

of the active compounds within the secretion, which may target particular bacterial components or pathways. Further investigation is needed to elucidate these mechanisms. The results of the HPLC fractionation suggest that specific compounds within the secretion are responsible for the antimicrobial activity. The fraction eluting at 5.3 minutes, which showed the most significant activity against *E. coli*, is a prime candidate for further investigation and compound identification.

The limited diffusion of activity observed in the disk diffusion assays could be attributed to the potentially high molecular weight of some of the active compounds in the leech secretion. Larger molecules diffuse more slowly through agar, which may limit their ability to reach and inhibit bacterial growth at a distance from the disk. This limitation of the disk diffusion method has been noted in other studies involving complex biological mixtures [14].

The protease activity detected in the zymography assay could play a role in the overall antimicrobial activity of the secretion. Proteases can potentially enhance the activity of antimicrobial peptides by, for example, cleaving bacterial proteins or increasing the permeability of bacterial membranes. Further studies are needed to explore the potential synergistic effects of proteases and other antimicrobial components in the *H. verbana* secretion [17].

The novelty of this study lies in the preliminary characterization of *H. verbana* leech secretions and the demonstration of their selective antimicrobial activity against specific multidrug-resistant bacteria. While the results are promising, further research is essential to isolate, identify, and characterize the active compounds, determine their precise mechanisms of action, and evaluate their potential for therapeutic use.

5. Conclusion

This study provides evidence that *H. verbana* leech secretions contain components with in vitro antimicrobial activity against *E. coli* and S. aureus. The preliminary characterization using HPLC identified a specific fraction with significant antimicrobial activity, highlighting the potential of this fraction for further investigation. These findings support the notion that *H. verbana* secretions may represent a valuable source of novel antimicrobial agents. Future research should focus on the isolation and identification of active compounds, elucidation of their mechanisms of action, and assessment of their in vivo efficacy and safety.

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References

1. Zhou, Y., Yu, Y., Huang, Q., Zheng, H., Zhan, R., Chen, L., & Meng, X. (2023). Simultaneous Determination of 26 Pesticide Residues in Traditional Chinese Medicinal Leeches by Modified QuEChERS Coupled with HPLC-MS/MS. ACS Omega, 8, 12404 12410.
2. Marquez-Gil, K.Y., Mesa, E., Kouka, N., Fonarov, I., & Casadesus, D. (2023). Medicinal Leeches to Aid in Post-procedural Hematoma Evacuation. *Cureus, 15*.
3. Diak, N., Śliwińska, M.A., Student, S., & Świątek, P. (2023). The three-dimensional conformation and activity of mitochondria in syncytial male germ line-cysts of medicinal leeches. *Cell and Tissue Research, 394,* 325 - 342.
4. Tong, Z., Song, L., Chen, S., Hu, J., Hou, Y., LIU, Q., Ren, Y., Zhan, X., & Zhang, Q. (2022). Hagfish-inspired Smart SLIPS Marine Antifouling Coating Based on Supramolecular: Lubrication Modes Responsively Switching and Self-healing Properties. *Advanced Functional Materials, 32*.
5. Xia, X., Li, M., Wei, R., Li, J., Lei, Y., & Zhang, M. (2023). Intracerebral hirudin injection alleviates cognitive impairment and oxidative stress and promotes hippocampal neurogenesis in rats subjected to cerebral ischemia. *Neuropathology, 43.*
6. Volkova, A., & Semenyuk, P.I. (2023). Tyrosine phosphorylation of recombinant hirudin increases affinity to thrombin and antithrombotic activity. Proteins: Structure, 92, 329 - 342.
7. Brørs, G., Gjeilo, K.H., Lund, T., Skevik, K., Aa, E., Høvik, L.H., Skarsvaag, T., & Mjølstad, O.C. (2023). Amiodarone-induced phlebitis: Incidence and adherence to a clinical practice guideline. *European journal of cardiovascular nursing.*
8. Cernuda Martínez, J.A., Suárez Mier, M.B., Martínez Ortega, M.D., Casas Rodríguez, R., Villafranca Renes, C., & Del Río Pisabarro, C. (2023). Risk factors and incidence of peripheral venous catheters-related phlebitis between 2017 and 2021: A multicentre study (Flebitis Zero Project). The journal of vascular access, 11297298231189963.
9. Rahmani, G., & O'Sullivan, G.J. (2023). Acute and chronic venous occlusion. The British journal of radiology, 20230242.
10. Wen, C., Chen, B., Lin, R., Dai, H., Tang, K., Zhang, G., Huang, J., Liao, C., Zeng, L., Xiang, X., Yang, J., & Huang, Y. (2022). Clinical Implications of Phenotypes of Hemodialysis Patients With Central Venous Occlusion or Central Venous Stenosis Defined by Cluster Analysis. *Frontiers in Cardiovascular Medicine, 9.*
11. Sonani, S., & Dudhamal, T.S. (2023). Leech therapy and adjuvant Ayurveda treatment in the management of diabetic foot ulcer with atherosclerosis. *BLDE University Journal of Health Sciences, 8*, 192 - 196.
12. Battin, A.O., Hobeika, N., & Zdilla, M.J. (2023). Systematic review of medicinal leech therapy in urology. *African Journal of Urology, 29*, 1-13.
13. Özkaya, D. (2023). Keratitis following leech therapy for periocular eczematous dermatitis: a case report. *BMC Complementary Medicine and Therapies, 23*.
14. Patel, J.B., Weinstein, M.P., Eliopoulos, G.M., Jenkins, S.G., Lewis, J., Limbago, B.M., Mathers, A.J., Mazzulli, T., Patel, R., Richter, S.S., Satlin, M.J., Swenson, J.M., Traczewski, M.M., Turnidge, J., & Zimmer, B.L. (2019). Performance standards for antimicrobial susceptibility testing.

15. CLSI. (2023). Performance Standards for Antimicrobial Susceptibility Testing. 34th ed. Clinical and Laboratory Standards Institute.
16. Lye, D.C., Kwa, A.L., & Chlebicki, P. (2011). World health day 2011: antimicrobial resistance and practical solutions. Annals of the Academy of Medicine, Singapore, 40 4, 156- 7.
17. Baskova, I.P., Kharitonova, O.V., & Zavalova, L.L. (2012). Lysozyme activity of the salivary gland secretion of the medicinal leeches H. verbana, *H. medicinalis*, and *H. orientalis*. Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 6, 138-143.
18. Vargas, N., Lieberman, B., Hwang, L.K., & Badner, V. (2023). Hirudotherapy for Venous Congestion in an Almost Completely Avulsed Earlobe without Microsurgical Repair. Plastic and Reconstructive Surgery Global Open, 11.