

Chemical Characterization of Soil Bacterial Extracts with Antimicrobial Activity Using High-Resolution Mass Spectrometry

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Abstract
Streptococcus pyogenes is a major human pathogen that causes several infections. Recently, it has become harder to control because it has become more resistant to common antibiotics. This fact has made people more interested in locating new natural sources of compounds that kill bacteria. Soil bacteria are a big part of modern microbial research because they can make bioactive secondary extracts. The goal of this study was to find and test the antimicrobial properties of several soil sours , with a focus on Streptococcus pyogenes, and to connect the chemical makeup of the bacterial extracts to their biological activity. We took soil samples from different places and used standard lab methods to isolate and purify bacterial strains. We used three common bioassay methods cross-line, disk diffusion, and beaker agar to test how well the antibacterial activity worked. There were clear differences between the isolates in terms of how well they inhibited growth. One isolate had higher and more stable activity than the others. High-resolution mass spectrometry (HRMS) analysis of the most potent extract showed that it contained several organic compounds from different chemical classes, such as lipids and heterocyclic compounds. These compounds are likely to help the extract fight bacteria. These findings suggest that soil bacteria could be a promising way to identify new natural compounds that could help make new treatments for the growing problem of bacterial resistance (Cunningham, 2000; Ventola, 2015; Bardi, 2012).

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Keywords: Soil bacteria, antimicrobial, HRMS, secondary metabolite, chemical composition

Introduction

Antimicrobial agents, which are specifically aimed at Streptococcus pyogenes, a clinically important human pathogenic bacterium. Exploration of antibacterial agents in soil samples Bacteria were isolated from various sites, and antibacterial potential of the isolated bacteria was warranted by performing on standard *in vitro* testing including cross-streak method, disc-diffusion assay and agar well diffusion assay. These methods were adopted to satisfy the inhibiting activities for searching bacterial isolates with the maximum antibacterial activity. Moreover, optimum bioactive compounds (secondary metabolites) were recovered from most active bacterial isolates using suitable organic solvents. Their chemical composition was subsequently confirmed by the high resolution mass spectrometry (HRMS). Other than that HRMS is just fantastic for complex organics. This unique and simple integration of bioassay and advanced chemical analysis helps to correlate antimicrobial activity with the molecular structure of bacterial extracts. This provides a solid scientific platform for researchers exploring new compounds that may well become drugs in the future. This work represents an innovative qualitative contribution to the screens

for bacteria-sourced natural antibiotic metabolites, since only a minor proportion of studies combine diverse assays and high resolution spectroscopy (one bacterium–S. pyogenes specificity), which may enhance both practical as well academic values from results achieved. Bacterial infections, at this very moment, are the largest health concern in the world. Considering the rapid spread in antibiotic resistant pathogenic bacteriaand this is a very alarming thought. The uncontrolled use of these antibiotics has led to the development of multidrug-resistant (MDR) strains, decreasing efficacy of the treatment options and increasing the morbi-mortality caused by infections (Ventola, 2015; WHO,WHO, 2023) [2, 11]. Clinical importance The Gram positive bacterium S. pyogenes is a pathogen that has very high relevance to medicine. In its link with a long list of diseases, we experience irritation over here or a minor infection over there (sore throat, skin lesions), right through to atheization Fe blunt trauma Logical Fallacies Misdirection-At this point im going to take you into another directionering your house.

The untreated simple sore throat caused by this type of bacteria used to be common in children but is now seldom seen thanks to the development of more effective antibiotics .Although for one of these infections penicillin is still taken as the first choice, the rising number of cases that do not immediately respond to an average treatment, and by some other alternatives will not go away either--both trends have given rise to ever greater concerns about whether current therapeutic methods are actually effective.

Streptococcus pyogenes, also known as Group A Streptococcus, is a Gram-positive bacterium of considerable clinical importance, as it is associated with a wide range of diseases, from uncomplicated throat and skin infections to serious conditions such as rheumatic fever, necrotizing fasciitis, and sepsis (Cunningham, 2000; Walker *et al*., 2014) [1, 10]. Although penicillin remains the standard treatment for these infections, the increasing number of treatment failures and the emergence of resistance to some alternative antibiotics have raised growing concerns about the effectiveness of current therapeutic options (Stevens *et al*., 2014) [7].

Antimicrobial resistance remains one of the most significant problems posing a serious threat to global health; however, the development of new antibiotics is a crucial area requiring further progress (Chmagh, Aalaa A., *et al*, 2023) [8]. In light of these challenges, recent research has focused on exploring natural sources, particularly microorganisms isolated from soil, as a rich reservoir of bioactive compounds with antibacterial activity. Soil bacteria, especially those genera that produce secondary metabolites, are a major source for antibiotic discovery, with estimates suggesting that a significant proportion of known antibiotics are derived from terrestrial microorganisms (Bérdy, 2012) [3]. Numerous studies have demonstrated that bacterial extracts possess broad-spectrum antimicrobial activity against pathogenic bacteria, including antibiotic-resistant strains, thanks to the diversity of their chemical compositions and multiple biological mechanisms of action (Newman & Cragg, 2020) [6]. Understanding the relationship between the biological activity of these extracts and their chemical composition is a crucial step in developing new therapeutic compounds of natural origin.Advanced analytical techniques, particularly high-resolution mass spectrometry (HRMS), play a fundamental role in characterizing complex organic compounds and determining the precise molecular formulas of bioactive secondary metabolites. This technique is widely used in natural product drug discovery studies due to its high accuracy and ability to analyze complex mixtures of compounds in biological extracts (Wolfender *et al*., 2019). Based on the foregoing, this study aims to isolate bacteria from different soil samples and evaluate their antibacterial activity against *Streptococcus pyogenes* using a range of biological assays. The active compounds in the most effective bacterial extracts will then be analyzed using high-resolution mass spectrometry to explore their potential as sustainable sources for developing new antibiotics capable of addressing the growing challenges of bacterial resistance. This approach is critical given that existing antibiotics are continuously facing novel resistance mechanisms developed by bacteria, often leading to treatment failures (Carrano *et al*., 2018, p. 136) [13]. The extensive use of antibiotics across human health, agriculture, and animal production has exacerbated this issue, contributing to the emergence of multi-resistant bacteria and a significant increase in mortality rates (Ardalani *et al*., 2021) [12]. The escalating global health crisis attributable to antibiotic-resistant infections, which accounted for 1.2 million deaths globally in 2019, underscores the urgent need for novel antimicrobial agents (Freezman *et al*., 2023, p. 1) [14]. This alarming trend is projected to worsen, with estimates indicating that by 2050, antimicrobial resistance could lead to 10 million deaths annually, surpassing cancer as a leading cause of mortality (Rammali *et al*., 2024, p. 2; Riyanti *et al*., 2020, p. 2) [15, 16]. The continuous and widespread abuse of antimicrobials in both medical and livestock settings has accelerated the evolution of pathogen resistance, leading to persistent and highly transmissible infectious diseases (Tenebro *et al*., 2021) [17].

Objective

The aim of this study was to evaluate the antibacterial activity of several soil bacterial isolates against Streptococcus pyogenes and to investigate a possible relationship between their biological activity and chemical composition using high-resolution mass spectrometry (HRMS).

Materials& Method

Table 1: Materials and apparatus used for bacterial isolation, extraction, and High-Resolution Mass Spectrometry analysis

Category	Material / Equipment	Purpose	Specifications / Grade
Culture media	Nutrient Agar	Isolation and purification of bacterial isolates	Microbiological grade
Culture media	Nutrient Broth	Cultivation of bacterial isolates and enhancement of secondary metabolite production	Microbiological grade
Culture media	Blood Agar	Cultivation of *Streptococcus pyogenes* and antibacterial activity assessment	Microbiological grade
Solutions	Physiological saline (0.85% NaCl)	Preparation of bacterial suspensions and serial dilutions	Sterile
Laboratory tools	Petri dishes, test tubes, micropipettes	Routine bacterial handling and culturing	Sterile
Biological material	Bacterial isolates	Production of bioactive secondary metabolites	Isolated from soil samples
Biological material	*Streptococcus pyogenes*	In vitro antibacterial assays	Reference strain
Organic solvents	Ethyl acetate	Extraction of secondary metabolites	Analytical grade
Organic solvents	Methanol	Sample preparation for HRMS analysis	HRMS grade
Separation tools	Glass separatory funnel	Liquid–liquid extraction	Standard laboratory glassware
Equipment	Rotary evaporator	Concentration of organic extracts under reduced pressure	Temperature-controlled
Analytical instruments	High-Resolution Mass Spectrometer (HRMS)	Molecular characterization of bioactive compounds	Equipped with ESI source
Filtration	0.22 µm membrane filter	Sample filtration prior to analysis	Sterile

1. Microorganism Isolation

Pure bacterial isolates collected from soil samples were cultured in nutrient broth to enhance the production of secondary metabolites. Pathogenic bacteria were cultured in a natural agar/broth medium (nutrient agar), and target bacteria were prepared for *in vitro* antibacterial assays. Sequential dilutions were performed in physiological saline (0.85% sodium chloride) to ensure purity, and all cultures were maintained under standard laboratory conditions.

2. Crude Extract Preparation

Secondary metabolites were isolated using a 1:1 volumetric ratio of ethyl acetate to bacterial culture. The mixture was thoroughly mixed, and the organic layer was separated. The extract was concentrated under reduced pressure using a rotary evaporator to obtain the crude extract. The dried extract was reconstituted in high-purity methanol to form a basic solution (1 mg/mL). It was then thoroughly mixed and filtered through a 0.22 µm membrane filter prior to high-resolution mass spectrometry (HRMS) analysis. All samples were stored at 4°C until analysis.

3. High-Resolution Mass Spectrometry (HRMS) Analysis

HRMS analysis was performed using an instrument equipped with an electron spray ionization source (ESI). Analyses were performed in both positive and negative ionization modes over a mass range of 100–1500 m/z. Instrument parameters were optimized to increase mass accuracy. HRMS data were used to estimate molecular formulas, and preliminary identification of compounds was made by comparing the results to a spectroscopic reference database.

4. Data Analysis

Our study involved analyzing chemical data from HRMS data as well as antimicrobial biological assays to identify compounds with potential activity against Streptococcus pyogenes. Compounds were grouped based on their chemical class, molecular weight, and peak quality (peak evaluation). This method allowed us to analyze the impact of chemical diversity on biological activity.

Results

1. Antimicrobial Activity of Bacterial Isolates Isolated from Soil

Bacterial isolates from soil samples showed significant antibacterial activity against *Streptococcus pyogenes* when tested using three different biological methods: the linear cross-sectional test, the disk diffusion test, and cup method (Table 2).

Table 2: Antibacterial activity of selected soil bacterial isolates against Streptococcus pyogenes

S.No	Isolation No	*Streptococcus pyogenes (mm) Line Cross M*	*Streptococcus pyogenes (mm) disc diffusion M*	*Streptococcus pyogenes (mm) cup M*
1	3	5	5	0
2	4	10	52	10

Clear differences in inhibition levels were observed among the tested isolates, indicating differences in their ability to produce secondary metabolites with antibacterial activity. Isolate 4 emerged as the strongest and most stable isolate across all testing methods, recording the largest inhibition zones compared to the other isolates.

The inhibition zone diameter for isolate 4reached 10 mm in the linear cross-sectional test, 50 mm in the disc diffusion test, and 10 mm in the cup method, confirming its potent multi-molecular antibacterial activity against *Streptococcus pyogenes* (Table 2).

In contrast, isolate 3 showed significantly lower antibacterial activity, with an inhibition zone diameter of 5 mm in both the cross-sectional and disc diffusion tests, while no inhibition zone was detected using the cup method. This indicates a lower efficiency in the production or release of the active compound compared to isolate 4.

2. **Chemical Analysis of Bacterial Extracts Using High-Resolution Mass Spectrometry**

High-resolution mass spectrometry (HRMS) analysis of bacterial extracts from the most virulent bacterial isolates revealed a complex and distinct chemical signature, identifying over 130 organic compounds (Table 3). Most compounds were highly similar to reference databases, with mass errors no greater than ±2 ppm, demonstrating the accuracy and reliability of the spectroscopic analysis.

Table 3: Selected high-confidence compounds identified in the bacterial extract by HRMS analysis

No.	Compound name	Molecular formula	Calculated MW (Da)	RT (min)	ΔMass (ppm)	Peak Rating	Annotation status
1	Oleamide	$C_{18}H_{35}NO$	281.27	18.45	-0.85	9.2	Full match
2	C16-Dihydroceramide	$C_{34}H_{69}NO_3$	543.53	22.30	-1.12	9.0	Full match
3	2,4-Hexadiynal	C_6H_4O	92.03	3.12	+0.45	8.7	Full match
4	Ceramide d18:1/20:0	$C_{38}H_{75}NO_4$	593.57	25.60	-1.05	9.4	Full match
5	Nitrogen-containing heterocycle (example)	$C_{12}H_{14}N_2S$	218.09	11.85	+0.62	8.6	Full match

Values represent the average diameter of the inhibition zone (mm).

The identified compounds belong to several chemical classes known for their antibacterial activity, including lipid compounds (such as oleimide and C16-dihydroxyceramide), heterocyclic compounds containing nitrogen and sulfur, and molecules rich in amine groups. These classes are associated with several inhibition mechanisms, including damage to bacterial cell membranes and interference with essential metabolic pathways.

3. Statistical Evaluation of High-Resolution Mass Spectrometry Data

3.1. Compound Classification

Statistical analysis revealed that 79.88% of the detected compounds were classified as perfect matches, demonstrating the quality of the molecular identification process (Table 4).

Table 4: Distribution of annotation status for the identified compounds.

Annotation status	Number	Percentage (%)
Full match	131	79.88
No match	17	10.37
No results	10	6.10
Not the top hit	6	3.66

Approximately 80% of the identified compounds showed a full match, indicating a high quality of chemical annotation and compound identification.

3.2. Mass Measurement Accuracy

The average mass deviation was -0.89 ppm, with a standard deviation of ±4.67 ppm, demonstrating the excellent accuracy of the high-resolution mass spectrometry system used (Table 5).

Table 5: Descriptive statistics of mass measurement accuracy (ppm)

Statistical parameter	Value
Mean	-0.89
Standard deviation	±4.67
Median	-1.44
Range	-17.86 to +17.15

*Negative Values Indicate That the Measured Masses Were Slightly Lower than the Theoretical Masses, with Moderate Variability and a Limited Number of Outliers

3.3. Molecular Weight Distribution

The molecular weights of the identified compounds ranged from 92.026 to 593.574 Da, with an average molecular weight of approximately 300.5 Da, reflecting the presence of both low-molecular-weight metabolites and structurally complex bioactive molecules (Table 6).

Table 6: Characteristics of molecular weight distribution of the identified compounds

Parameter	Value
Minimum MW	92.026 Da
Maximum MW	593.574 Da
Mean	~300.5 Da
Median	~289.5 Da

*This distribution reflects the presence of both low-molecular-weight compounds and more complex molecules, such as ceramides and bioactive lipids.

3.4. Chromatographic Retention Time Characteristics

Retention times spanned from 0.17 to 29.97 min, with a mean value of ~14.5 min, indicating substantial diversity in compound polarity and chromatographic behavior (Table 7).

Table 7: Statistical characteristics of retention time (min)

Parameter	Value
Range	0.17 – 29.97
Mean	~14.5
Median	~14.4

*The wide retention time range indicates diversity in compound polarity, with a pronounced clustering of compounds eluting in the mid-retention time region.

3.5. Peak Intensity and Quality Metrics

Peak area analysis revealed an approximately 1000-fold variation between minimum and maximum signal intensities, suggesting marked differences in compound abundance and/or ionization efficiency (Table 8). Additionally, most detected features exhibited moderate to high peak quality, with a median peak rating of ~7.8, confirming the robustness of the HRMS data (Table 98).

Table 8: Peak area range of the identified compounds

Parameter	Value
Minimum area	1.63×10^6
Maximum area	1.64×10^9
Relative difference	~1000-fold

*This large variation in signal intensity indicates marked differences in relative abundance and/or ionization efficiency among the detected compounds.

4. Integration of Antibacterial Activity and HRMS Findings

Integrating the results of antibacterial biological tests (Table 3) with the results of HRMS analysis (Tables 2 and 4-9) revealed a clear correlation between the strength of antibacterial activity and the chemical diversity of the bacterial extract. The most potent isolates were associated with a variety of lipid compounds and heterocyclic compounds, which are known for their antibacterial properties.

Table 9: Descriptive statistics of peak rating

Parameter	Value
Mean	~6.9
Median	~7.8
Range	1.0 – 10.0

*Peak rating values ranged from 1.0 to 10.0, with a mean of ~6.9 and a median of ~7.8, indicating that most detected peaks exhibited good to high signal quality

These results suggest that the strong inhibitory effect against Streptococcus pyogenes is likely due to the synergistic effect of a group of active compounds rather than a single compound, thus enhancing the pharmacological value and potential use of bacterial extracts as a sustainable source for the development of new antibiotics.

Conclusions

Our study demonstrates that multiple resources can be leveraged to develop antimicrobials. Soil bacteria are a promising and sustainable source for bioactive compounds with antibacterial activity against Streptococcus pyogenes. The bacterial isolates studied showed significant variation in their inhibitory potency, indicating differences in their metabolic ability to produce bioactive secondary compounds. Furthermore, high-resolution mass spectrometry analysis revealed a high degree of chemical diversity in bacterial extracts, supporting a link between molecular structure and antibacterial activity. These results highlight the importance of soil as a largely untapped natural reservoir for the discovery of potential alternatives to conventional antibiotics and provide a scientific basis for further in-depth future studies in the field of antibacterial agent development.

Recommendations

Future studies should be conducted to isolate and purify single active compounds from extracts of the most potent isolates and to investigate their chemical structures in detail.
Evaluate the molecular mechanisms underlying the antibacterial activity of the discovered compounds using advanced molecular biology techniques.
Study the biological activity of bacterial extracts against a wide range of pathogenic bacterial strains, including antibiotic-resistant strains.
Conduct cytotoxicity and biosafety tests to assess the potential for practical use of these compounds.
Promote the integration of modern bioanalytical techniques, such as HRMS, with traditional bioassays to accelerate the discovery of new compounds of interest for medical use.
Support research exploring microbial diversity in different natural environments as a strategic source for the discovery of new bioactive compounds.

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