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Research Paper

Isolation and Antibacterial Properties of Actinomycetes from Licorice (*Glycyrrhiza glabra*)

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ABSTRACT

Introduction: Actinomycetes associated with the medicinal plant licorice (*Glycyrrhiza glabra*) were investigated for their potential to produce novel antibiotics, an area of growing importance in combating bacterial resistance.

Materials & Methods: In this study, a total of 75 actinomycete isolates were obtained from licorice plant samples collected in Ilam Province, Iran. These samples were carefully selected due to licorice's traditional use in herbal medicine, suggesting a rich microbial diversity. **Results:** Molecular identification through *16S rRNA* gene amplification confirmed that 57 of the isolates belonged to the class Actinomycetia, within the phylum Actinomycetota. Further screening for biosynthetic gene clusters (BGCs) revealed that an impressive 96% of the isolates harbored genes for nonribosomal peptide synthetases (NRPS). In contrast, only 28% and 17% of the isolates contained genes associated with polyketide synthase type I (PKS-I) and type II (PKS-II), respectively. Utilizing agar well diffusion assays, the study demonstrated that 16 isolates (28%) exhibited significant antibacterial activity against both drug-resistant and drug-sensitive strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Among these, two isolates, S12 and S14, showcased remarkable broad-spectrum antibacterial properties by inhibiting three members of the ESKAPE pathogen group.

Conclusion: The strong correlation between the presence of NRPS genes and antibacterial activity underscores the potential of actinomycetes associated with licorice as a promising source of novel antimicrobial compounds. These findings emphasize the importance of bioprospecting medicinal plant-derived microbiomes as a strategic approach to address the escalating global challenge of antibiotic resistance, paving the way for future research and development in antimicrobial therapies. Future research should focus on elucidating the genetic and metabolic networks that underpin these interactions to fully exploit their pharmaceutical potential.

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1. Introduction

Antibiotics have been a cornerstone of modern medicine since their discovery, drastically reducing the burden of bacterial diseases and transforming clinical outcomes worldwide. By offering effective treatment options for bacterial infections, antibiotics have significantly lowered morbidity and mortality rates and are considered one of the greatest medical achievements of the 20th century [1]. Despite these successes, the benefits of antibiotics are being increasingly undermined by the rise of antibiotic-resistant bacteria, a phenomenon exacerbated by the overuse, misuse, and inappropriate prescription of these life-saving drugs. These practices have created an environment in which bacteria can evolve resistance mechanisms, rendering many commonly used antibiotics ineffective.

The proliferation of multidrug-resistant (MDR) pathogens, including *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, has emerged as a significant public health challenge. These pathogens are associated with increased morbidity, mortality, and healthcare costs, particularly in hospital settings where they complicate infection management and treatment outcomes [2]. The situation is further aggravated in low- and middle-income countries, where limited access to effective infection control measures and antibiotic stewardship programs contribute to the unchecked spread of resistance [3]. This crisis demands a multifaceted response, including the development of novel therapeutic strategies, optimization of antibiotic usage, and exploration of alternative approaches to bacterial infection management.

Among the promising avenues to counteract antibiotic resistance is the discovery of new antibiotics, particularly those derived from natural sources. Historically, natural products have served as the foundation for most antibiotics, with microorganisms such as actinomycetes playing a critical role in their discovery and development. For instance, members of the genus *Streptomyces* within the phylum Actinomycetota are responsible for producing over two-thirds of clinically used antibiotics, demonstrating their unparalleled biosynthetic capabilities [4]. These filamentous, gram-positive bacteria possess specialized gene clusters, such as nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), that encode enzymes capable of synthesizing a diverse array of bioactive compounds. This genetic machinery allows for the modular assembly of complex molecules with significant antimicrobial, antifungal, and anticancer properties, making actinomycetes indispensable in pharmaceutical development [5].

Genomics, metabolomics, and synthetic biology advancements have further expanded the potential for discovering novel antibiotics. By unlocking the biosynthetic potential of actinomycetes and activating cryptic gene clusters—those that remain silent under standard laboratory conditions—researchers are uncovering previously untapped reservoirs of bioactive compounds. These breakthroughs are critical for addressing the urgent need for new antimicrobial agents capable of overcoming resistance mechanisms and combating MDR pathogens [6].

Medicinal plants represent another promising source of novel antibiotics due to their symbiotic relationships with endophytic microorganisms. Licorice (*Glycyrrhiza glabra*), a medicinal plant widely recognized for its pharmacological properties, including anti-inflammatory, antimicrobial, and antioxidant effects, serves as a reservoir for endophytes such as actinomycetes [7]. These endophytic communities contribute to the plant's therapeutic potential by producing bioactive secondary metabolites and enhancing the synthesis of key compounds such as glycyrrhizin [8]. Additionally, endophytes improve the plant's resilience to abiotic stresses, highlighting the intricate ecological interactions that can be harnessed for drug discovery [9].

Given the critical role of actinomycetes and their association with medicinal plants, this study focuses on exploring the biosynthetic capabilities of actinomycetes isolated from *G. glabra*. By leveraging advanced genomic and metabolomic approaches, we aim to identify novel bioactive compounds with antimicrobial potential. This research seeks to address the escalating challenge of antibiotic resistance and contribute to the discovery of new therapeutics that are essential for safeguarding global health.

2. Materials and Methods

2.1. Sample collection and isolation of endophytic actinomycete

Licorice (*G. glabra*) plant samples were collected in the spring of 2023 from various regions of Ilam Province, Iran. These samples were stored in sterile plastic bags and transported to the laboratory on ice to preserve microbial integrity.

In the laboratory, the plant samples underwent a modified six-step surface sterilization process within 24 hours of collection, as described elsewhere [10]. The sterilized plant parts—roots, stems, and leaves—were aseptically

fragmented into 1-centimeter pieces and spread on starch casein agar (SCA), which was supplemented with cycloheximide (50 µg/mL) and nalidixic acid (20 µg/mL) to inhibit the growth of fungi and non-actinomycete bacteria, respectively [11]. The culture media were incubated at 28 ˚C for up to four weeks, with regular observations made for the potential growth of new colonies. The putative actinomycete colonies were purified through repeated streaking on the international streptomyces project 2 (ISP2) medium. Additionally, 100 µL of the final rinse solution was applied to SCA plates and incubated at 28 ºC for two weeks to assess microbial growth and the effectiveness of the surface sterilization process.

2.2. DNA Isolation and molecular identification of actinomycetes

Genomic DNA extraction were conducted for all endophytic isolates using a straightforward boiling method, as outlined in previous studies, followed by polymerase chain reaction (PCR) with taxon-specific primers (Table 1) to identify actinomycetes, as previously demonstrated [15].

2.3. Evaluation of antibacterial activity of actinomycetes

Each actinomycete isolate was cultured in trypticase soy broth (TSB) and ISP2 medium at 28 ˚C while being shaken at 180 rpm. After 7 and 14 days of cultivation, the fermentation broth was centrifuged at 13,000×g for 15 minutes to remove biomass. An equal volume of ethyl acetate was then added to the supernatant and shaken vigorously. Following this, a vacuum rotary evaporator was used to evaporate the organic layer at 40 ˚C. The resulting organic extracts were employed for antimicrobial activity screening.

The drug-sensitive and resistant bacteria, as selective members of the ESKAPE *(Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp.) pathogens [16], were used to assess the antibacterial activity of the actinomycete strains (Table 2). These bacteria were grown overnight at 37 ˚C in Mueller-Hinton (MH) broth, which was subsequently adjusted to a 0.5 McFarland standard turbidity.

Bacterial lawns were prepared on MH agar, making wells approximately 6 mm in diameter using a sterilized cork borer. One hundred microliters (µL) of the crude extracts were added to each well. The plates were left at room temperature for one hour to allow the crude extract to diffuse before being incubated at 37 ˚C. After 24 hours, the diameters of the inhibition zones were measured in millimeters (mm). The control used was 100 µL of ethyl acetate.

2.4. Detection of *PKS-I, PKS-II,* and *NRPS* genes

Genes encoding NRPS and polyketide synthases I and II (PKS-I and PKS-II) were detected via PCR using specific primers (Table 1). Amplifications were performed with 30 cycles of denaturation (95 ˚C, 1 minute), annealing (58 ˚C or 60 ˚C, 1 minute), and extension (72 ˚C, 1 minute). Products were analyzed on 1.5% agarose gels.

Table 1. Oligonucleotide primers used in this study

Primer Name	Sequence (5'-3')	Gene	Product Size (bp)	Ref.
ACT235f	CGCGGCCTATCAGCTTGTTG	*16S rRNA*	640	[12]
ACT878r	CCGTACTCCCCAGGCGGGG			
A3F	GCSTACSYSATSTACACSTCSGG	*NRPS*	700-800	[13]
A7R	SASGTCVCCSGTSCGGTAS			
KIF	TSAAGTCSAACATCGGBCA	*PKS-I*	1200-1400	[14]
M6R	CGCAGGTTSCSGTACCAGTA			
PKS-II-A	TSGCSTGCTTCGAYGCSATC	*PKS-II*	600	[13]
PKS-II-B	TGGAANCCGCCGAABCCGCT			

3. Results

3.1. Isolation and morphological characterization of endophytic actinomycetes

Ninety-six bacterial isolates were obtained from licorice plants, of which 75 actinomycete isolates were ientified by morphology and gram staining. Actinomycete colonies exhibited powdery or chalky textures, firm and sticky structures, and pigmentation in white, orange, or gray, accompanied by a characteristic earthy odor.

3.2. Molecular identification of actinomycetes

PCR amplification of the *16S rRNA* gene successfully identified 57 isolates as actinomycetes, with amplicons of approximately 640 bp (Figure 1). The distribution of isolates among plant parts was as follows: 25 from stems (43.9%), 23 from roots (40.4%), and nine from leaves (15.8%).

3.3. Antibacterial activity of actinomycetes

Of the 57 molecularly confirmed isolates, 16(28%) exhibited antibacterial activity against the test pathogens. Seven isolates inhibited drug-resistant *S. aureus*, eight inhibited drug-sensitive *S. aureus*, and nine inhibited *P. aeruginosa*. Isolates S12 (stem) and S14 (root) showed broad-spectrum activity, inhibiting all three tested pathogens (Figure 2).

3.4. Detection of biosynthetic gene clusters (BGCs)

PCR revealed NRPS genes in 96% of isolates, PKS-I genes in 28%, and PKS-II genes in 17% (Figures 3, 4, and 5). Isolates S12 and S14, which exhibited the broadest antibacterial spectra, contained NRPS genes but lacked PKS genes, suggesting a strong correlation between NRPS clusters and bioactivity.

3.5 Summary of antibacterial activity and gene presence

The presence of *NRPS* and *PKS-I* genes was associated with higher antibacterial activity. Among the 16 active isolates, 62.5% inhibited one pathogen, 25% inhibited two pathogens, and 12.5% (isolates S12 and S14) inhibited three pathogens.

4. Discussion

This study highlights the significant potential of licorice (*G. glabra*) as a valuable source of bioactive actinomycetes, focusing on their ability to produce secondary metabolites through *NRPS* and *PKS-I* genes. These BGCs are crucial for synthesizing diverse bioactive compounds, many of which exhibit strong antibacterial properties. Our findings support previous research emphasizing the therapeutic promise of actinomycetes from plant-associated environments, particularly in drug discovery and pharmaceutical development [17].

The strong correlation between the detection of *NRPS* and *PKS-I* genes and the observed antibacterial activities underscores the essential role of actinomycetes in the biosynthesis of antimicrobial agents. These results reinforce the hypothesis that actinomycetes associated with licorice produce unique bioactive compounds capable of combating a wide range of bacterial pathogens. This is especially relevant in addressing the growing challenge of antibiotic resistance, as new compounds with distinct modes of action are urgently needed [18].

In addition to antibacterial properties, actinomycetes have diverse therapeutic applications. Secondary metabolites produced by these microorganisms also exhibit antifungal, anticancer, and immunomodulatory activities, significantly expanding their pharmaceutical potential [19]. actinomycetes from underexplored environments, such as licorice, represent an untapped reservoir for discovering molecules that could lead to breakthroughs in medical treatments.

Licorice plants create a unique microenvironment that promotes the growth, diversity, and metabolic capabilities of endophytic actinomycetes. This environment is characterized by a consistent supply of nutrients, specific metabolites, and physiological conditions conducive to

Table 2. ESKAPE pathogen members included in this study for evaluating antibacterial activity

Bacteria	Drug-sensitive	Drug-resistant
S. aureus	ATCC 25923	ATCC 33591
K. pneumoniae	ATCC 10031	ATCC 700603
P. aeroginosa	ATCC 27853	ATCC 2774
A. baumannii	ATCC BAA-747	

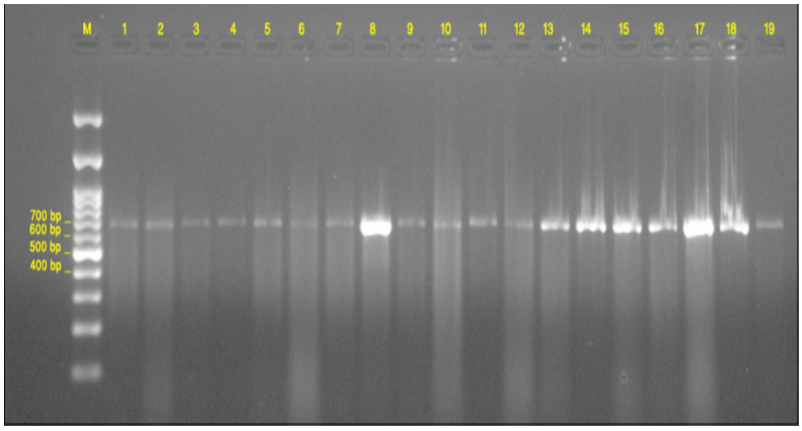

Figure 1. Agarose gel electrophoresis of *16S rRNA* PCR products from bacterial isolates

Note: Lanes M: DNA size marker; Lanes 1–19: PCR products from bacterial isolates showing a band at 640 bp, representing the amplified *16S rRNA* gene

microbial colonization and activity. Actinomycetes associated with licorice utilize these plant-derived compounds as substrates, enabling them to produce a range of secondary metabolites with potential therapeutic value [19].

The co-evolutionary relationship between licorice and its endophytic actinomycetes is a fascinating aspect of their interaction. Through long-term associations, these microorganisms have developed the ability to synthesize compounds that benefit both themselves and their plant hosts. Such compounds include growth-promoting phytohormones and antimicrobial agents that protect plants from pathogens, thereby enhancing plant resilience and productivity [20].

This co-evolutionary adaptation not only highlights the ecological importance of licorice but also positions it as an ideal candidate for bioprospecting. The unique biochemistry of the plant shapes the diversity and specialization of its microbial inhabitants, potentially leading to the discovery of novel bioactive compounds [21].

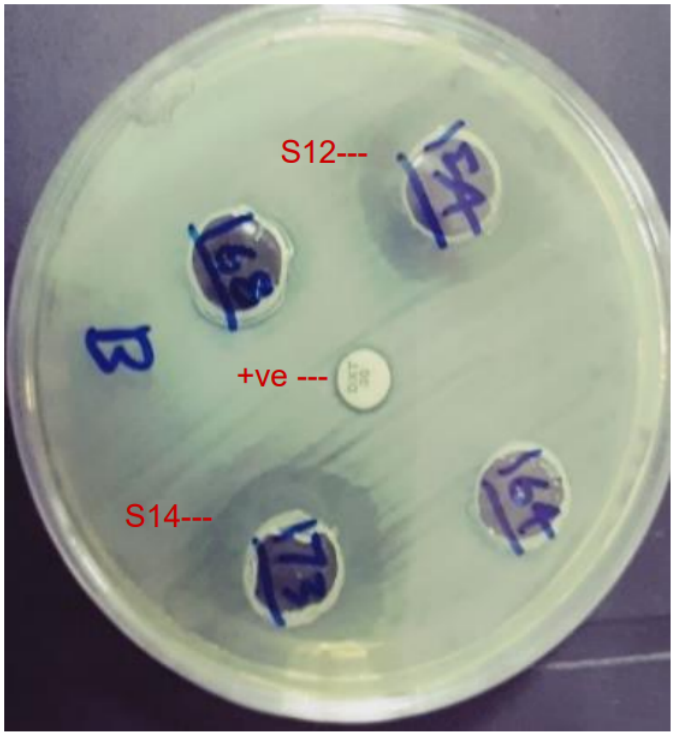

Figure 2. Antibacterial activity of isolates S12 and S14 against *S. aureus* (ATCC 33591)

Note: +ve: Positive control. Doxycycline disk (30 µg) placed in the center of the MH medium.

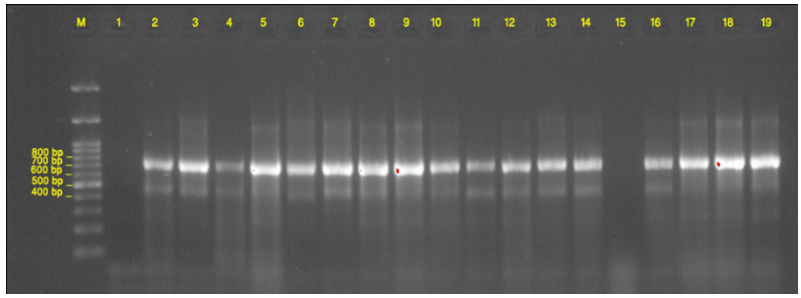

Figure 3. Agarose gel electrophoresis of NRPS gene PCR products from actinomycete isolates

Note: Lanes M: DNA size marker; Lane 1: Negative control; Lanes 2–19: PCR products from actinomycete isolates, displaying bands between 700–750 bp, indicative of the amplified *NRPS* gene.

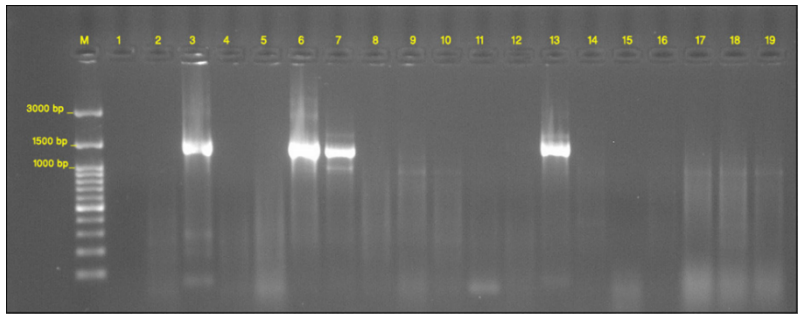

Figure 4. Agarose gel electrophoresis of *PKS-I* gene PCR products from actinomycete isolates

Note: Lanes M: DNA size marker; Lane 1: Negative control; Lanes 2–19: PCR products from actinomycete isolates showing a band at approximately 1200 bp, representing the amplified *PKS-I* gene.

Understanding plant-microbe interactions is vital for optimizing antibiotic discovery and other therapeutic advancements. The symbiotic relationship between licorice and its endophytic actinomycetes involves complex signaling pathways that regulate microbial activity and plant defense mechanisms. For example, the microbial synthesis of phytohormones, such as auxin, can influence plant growth and development while also indirectly affecting the production of microbial secondary metabolites [22]. These interactions highlight the intricate interdependencies that contribute to the biosynthetic potential of microbial communities.

The evolutionary dynamics of plant-microbe interactions reinforce their value in biotechnological applications. Beneficial microbes such as actinomycetes enhance plant resilience against environmental stresses and pathogens. This mutualistic relationship creates favorable conditions for microbial metabolite production, providing dual benefits for the agriculture and pharmaceutical industries [23]. By leveraging these interactions, researchers can identify novel biosynthetic pathways and optimize conditions for metabolite production, thereby improving the yield and efficacy of antibiotics [24].

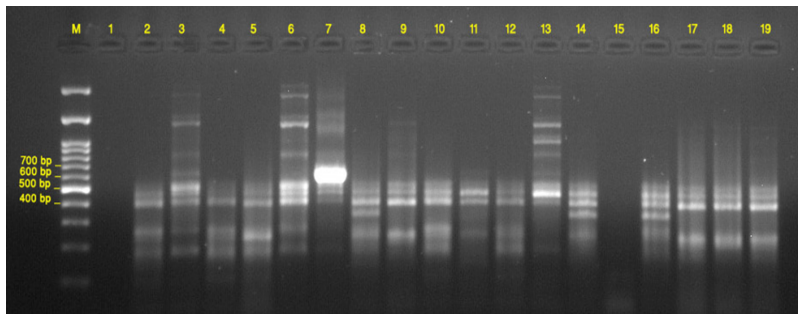

Figure 5. Agarose gel electrophoresis of *PKS-II* gene PCR products from actinomycete isolates

Note: Lanes M: DNA size marker; Lane 1: Negative control; Lanes 2–19: PCR products from actinomycete isolates exhibiting a band at 600 bp, corresponding to the amplified *PKS-II* gene.

The remarkable diversity of endophytic actinomycetes found in licorice emphasizes its immense potential for bioprospecting. Medicinal plants such as licorice serve as reservoirs of microbial biodiversity, often housing rare or unique strains with specialized metabolic capabilities. Exploring this diversity can lead to the identification of novel compounds with therapeutic applications, particularly those with antibacterial, antifungal, and anticancer properties [25].

Advances in genomic, transcriptomic, and metabolomic technologies provide powerful tools for bioprospecting. By examining the genetic and metabolic profiles of actinomycetes associated with licorice, researchers can identify BGCs to enhance drug discovery efforts.

5. Conclusion

In summary, licorice-associated actinomycetes represent a rich and underexplored resource for discovering bioactive compounds with significant therapeutic and agricultural potential. The presence of *NRPS* and *PKS-I* genes highlights their role in secondary metabolite production, particularly antibacterial agents. Licorice's unique microenvironment supports the metabolic versatility of these microorganisms, while plant-microbe interactions provide valuable insights into optimizing antibiotic discovery. Advancing our understanding of these interactions, together with the application of cutting-edge biotechnological tools, could unlock new frontiers in bioprospecting and drug development, ultimately contributing to human health and environmental sustainability.

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Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Data availability

The data supporting this study are not publicly available but are available from the corresponding author upon reasonable request.

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Authors' contributions

Conceptualization, study design, and statistical analyses: Sara Sadeghian and Fazel Pourahmad; Samples collections, experiments, data interpretation and writing: All authors; Final approval: Fazel Pourahmad and Mostafa Nemati.

Conflict of interest

The authors declared no conflict of interest.

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