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

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Abstract

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. 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. Molecular identification through 16S rRNA gene amplification confirmed that 57 of the isolates belonged to the Class *Actinomycetes*, of the phylum *Actinomycetota*.

Further screening for biosynthetic gene clusters (BGC) revealed that an impressive 96% of the isolates harbored genes for non-ribosomal 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.

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 underpinning these interactions to fully exploit their pharmaceutical potential.

٤٤ **Keywords:** Isolation, *Actinomycetes*, Endophytes, Licorice (*Glycyrrhiza glabra*), Antibacterial

٤٥ activity.

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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 lower 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 where 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 contributes 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 *Actinomycetota* phylum 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 non-ribosomal 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 like 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 *Glycyrrhiza*

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 essential for safeguarding global health.

2. Materials and Methods

2.1 Sample Collection and Isolation of Endophytic *Actinomycete*

Licorice (*Glycyrrhiza glabra*) plant samples were collected in spring 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 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 28ºC for up to four weeks, with regular observations made for the potential growth of new colonies. The putative actinomycetal 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 28ºC 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 extractions 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).

Table 1. List of oligonucleotide primers used in the study

Primer name	Sequence (5'-3')	Gene	Product size (bp)	Reference
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			

2.3 Evaluation of Antibacterial Activity of *Actinomycete*

Each actinomycetal isolate was cultured in trypticase soy broth (TSB) and ISP2 media at 28°C while being shaken at 180 rpm. After 7 and 14 days of cultivation, the fermentation broth was centrifuged at 13000 × 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 ESKPE pathogens (16), were used to assess the antibacterial activity of the actinomycetal strains (Table 2). These bacteria were grown overnight at 37ºC 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 37ºC. After 24 hours, the diameters of the inhibition zones were measured in millimeters (mm). The control used was a 100 µL volume of ethyl acetate.

Table 2. Members of ESKAPE pathogens included in the study for evaluating antibacterial activity

Bacteria	Drug-sensitive	Drug-resistant
Staphylococcus aureus	ATCC 25923	ATCC 33591
Klebsiella pneumoniae	ATCC 10031	ATCC 700603
Pseudomonas aeroginosa	ATCC 27853	ATCC 2774
Acinetobacter baumannii	ATCC BAA-747	

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

Genes encoding non-ribosomal peptide synthetases (*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.

3. Results

3.1 Isolation and Morphological Characterization of Endophytic Actinomycete

Ninety-six bacterial isolates were obtained from licorice plants, of which 75 actinomycete isolates were diagnosed by morphology and Gram staining. Actinomycetal 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 by plant part was as follows: 25 from stems (43.9%), 23 from roots (40.4%), and 9 from leaves (15.8%).

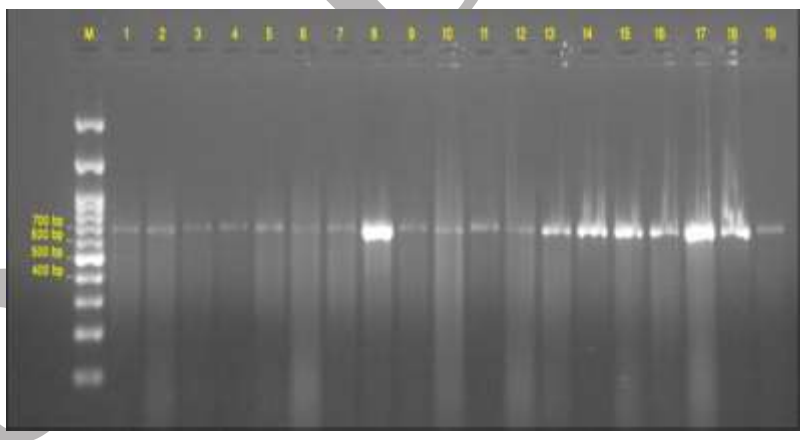

Figure 1. Agarose gel electrophoresis of 16S rRNA PCR products from bacterial isolates.

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

3.3 Antibacterial Activity of *Actinomycetes*

Of the 57 molecularly confirmed isolates, 16 (28%) exhibited antibacterial activity against 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).

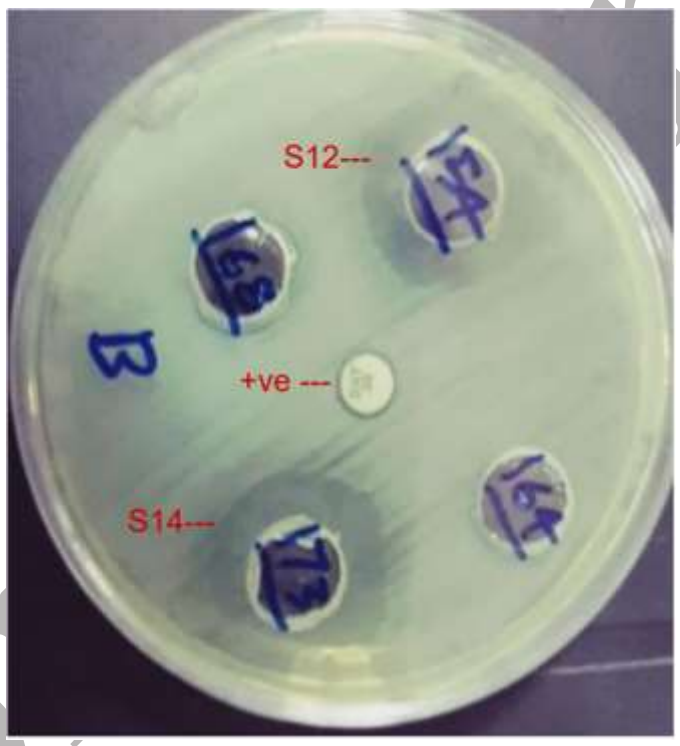

Figure 2. Antibacterial activity of the isolates S12 and S14 against *S. aureus* (ATCC 33591). +ve (Positive control), Doxycycline disk (30 µg) in the center of the MH medium.

3.4 Detection of Biosynthetic Gene Clusters

PCR revealed *NRPS* genes in 96% of isolates, *PKS-I* genes in 28%, and *PKS-II* genes in 17% (Figures 3-5). 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.

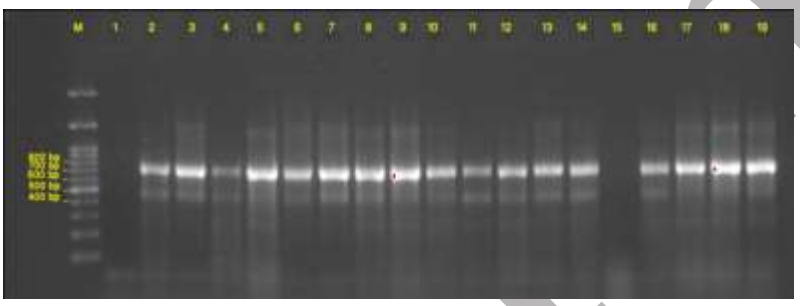

Figure 3. Agarose gel electrophoresis of *NRPS* gene PCR products from actinomycete isolates.

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

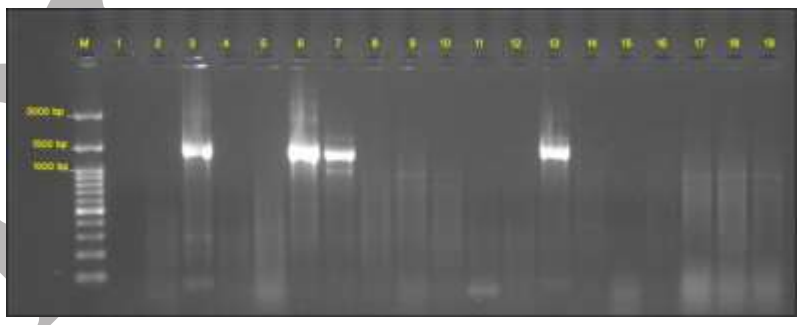

Figure 4. Agarose gel electrophoresis of *PKS-I* gene PCR products from actinomycete isolates.

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

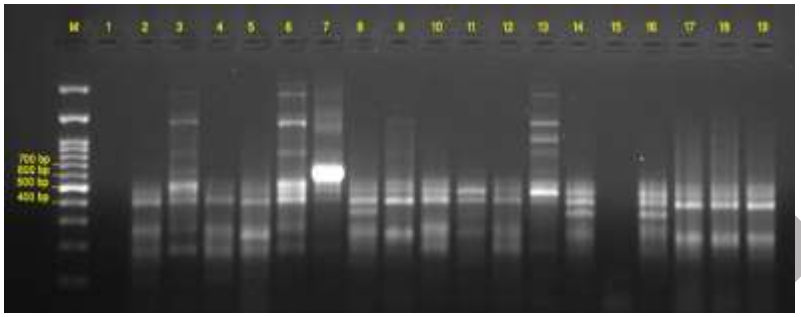

Figure 5. Agarose gel electrophoresis of *PKS-II* gene PCR products from actinomycete isolates.

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

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% (S12 and S14) inhibited three pathogens.

4. Discussion

This study highlights the significant potential of licorice (*Glycyrrhiza 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 have strong antibacterial properties. Our findings support previous research that emphasizes 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 tackling 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 like 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 microbial colonization and activity. 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, enhancing both their 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).

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 like *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).

The remarkable diversity of endophytic *Actinomycetes* found in licorice emphasizes its immense potential for bioprospecting. Medicinal plants like 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* linked to licorice, researchers can identify BGCs to enhance drug discovery efforts.

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 and the application of cutting-edge biotechnological tools could unlock new frontiers in bioprospecting and drug development, ultimately contributing to human health and environmental sustainability.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethics

This paper does not involve any research related to experimental animals or specific human diseases.

Data availability

The data supporting this study are not publicly available but can be shared upon reasonable request to the corresponding author.

Authors contribution

S. S. and F. P. proposed and designed the research, S.S., F. P., and M. N. collected samples S. S., F. P., and M. N. analyzed and interpreted data, S. S., F. P., and M. N. drafted the manuscript, S. S. and F. P. performed statistical analyses, F. P. and M. N. proved the final version of the manuscript

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