Isolation and Antibacterial Properties of *Actinomycetes* from Yellow Olive Tree (*Olea europaea*)

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Abstract

The symbiotic relationships between Actinomycetes and their host plants further enhance their potential as sources of bioactive compounds. These bacteria produce a wide array of secondary metabolites with antimicrobial, insecticidal, and anticancer properties, making them valuable for bioprospecting in pharmaceuticals and agriculture.

The ineffectiveness of existing antibiotics has resulted in higher morbidity and mortality rates, alongside escalating healthcare costs due to treatment failures. The rise of multidrug-resistant (MDR) pathogens poses a significant threat to global health, necessitating the discovery of novel antimicrobial agents. This study isolates and characterizes endophytic *Actinomycetes* from the yellow olive tree (*Olea europaea*), a plant known for its rich phytochemical composition, to evaluate their antibacterial potential against ESKAPE pathogens. Samples were collected from olive tree roots, and 54 bacterial isolates were obtained, with 45 (83.3%) identified as *Actinomycetes* through 16S rRNA gene amplification. Among these, 16 isolates (35.6%) exhibited antibacterial activity against drug-sensitive and drug-resistant strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Molecular screening revealed that 66.7%, 28.9%, and 93.3% of the isolates harbored non-ribosomal peptide synthetase (*NRPS*), polyketide synthase I (*PKS-I*), and polyketide synthase II (*PKS-II*) genes, respectively, which are associated with the biosynthesis of secondary metabolites. However, no direct correlation was found between these biosynthetic genes and antibacterial activity, suggesting that gene expression and environmental factors play crucial roles in metabolite production. The study highlights the potential of endophytic *Actinomycetes* from *Olea europaea* as a source of novel antimicrobial compounds, particularly in the fight against MDR pathogens. These findings underscore the

importance of exploring plant-associated microbes for developing new therapeutic agents to address the global antibiotic resistance crisis.

Keywords: Endophytic *Actinomycetes*, *Olea europaea*, Antibacterial activity, Biosynthetic gene clusters, Secondary metabolites

1. Introduction

The global health landscape is increasingly threatened by the rise of multidrug-resistant (MDR) pathogens, a crisis fueled by the overuse and misuse of antibiotics. This phenomenon has led to the emergence of "superbugs," pathogens resistant to multiple conventional antibiotics, making infections increasingly difficult to treat and pose a significant challenge to public health systems worldwide (1). The World Health Organization (WHO) has identified these MDR pathogens as critical threats, underscoring the urgent need for innovative antimicrobial strategies to combat this escalating crisis (2).

The ineffectiveness of existing antibiotics has resulted in higher morbidity and mortality rates, alongside escalating healthcare costs due to treatment failures (3). In response, researchers are exploring alternative therapies and novel antimicrobial agents, particularly from natural sources, to address the growing threat of antibiotic resistance. Recent advancements in natural product screening, marine-derived compounds, hybrid molecules, and microbial isolates have shown promise in identifying new antimicrobial agents (4). These efforts highlight the importance of leveraging natural biodiversity in the search for effective treatments against resistant pathogens.

Among the most promising sources of novel antimicrobial compounds are the *Actinomycetes*, a phylum of gram-positive bacteria renowned for their prolific production of secondary metabolites,

including antibiotics and bacteriocins. These bacteria, particularly those within the genus *Streptomyces*, are responsible for producing approximately two-thirds of all known natural antibiotics, making them invaluable in the fight against antibiotic resistance (5). *Actinomycetes* are ecologically versatile, thriving in diverse environments such as soil, marine ecosystems, and plant tissues, where they contribute to nutrient cycling, secondary metabolite production, and plant health (6). The production of these bioactive compounds is largely governed by biosynthetic gene clusters (BGCs), which encode the enzymatic machinery required for synthesizing secondary metabolites with diverse biological activities, including antibacterial, antifungal, and anticancer properties. The presence and diversity of BGCs in *Actinomycetes* are strongly correlated with their antimicrobial potential, as these gene clusters enable the production of structurally complex and functionally potent compounds that can target resistant pathogens (7).

The symbiotic relationships between *Actinomycetes* and their host plants further enhance their potential as sources of bioactive compounds. These bacteria produce a wide array of secondary metabolites with antimicrobial, insecticidal, and anticancer properties, making them valuable for bioprospecting in pharmaceuticals and agriculture (8). Endophytic *Actinomycetes*, in particular, have been shown to promote plant growth, protect against pathogens, and produce antibacterial compounds, highlighting their potential applications in sustainable agriculture and medicine (9).

Given the medicinal significance of the yellow olive tree (*Olea europaea*) and its rich phytochemical composition, this study focuses on isolating and characterizing endophytic *Actinomycetes* from *Olea europaea*. The research aims to evaluate the antibacterial properties of these *Actinomycetes* against a panel of pathogenic bacteria, including drug-resistant strains, thereby contributing to the ongoing search for novel antimicrobial agents. By exploring the synergistic potential of *Olea europaea* and its associated *Actinomycetes*, this study seeks to

advance our understanding of natural sources of antibiotics and their applications in combating MDR pathogens.

2. Materials and Methods

2.1 Sample Collection and Isolation of *Actinomycetes*

In this study, which was conducted in 2023, ten yellow olives (*Olea europaea*) saplings were obtained from the greenhouse of Ilam University, Iran, and transferred to the Microbiology laboratory.

In the laboratory, plant samples underwent a modified six-step surface sterilization procedure within 24 hours, as described in previous research (10). This involved washing with tap water for 10 minutes to remove soil and contaminants, followed by sterile separation of root, stem, and leaf tissues. The tissues were sequentially sterilized using 70% ethanol (1 minute), 5% sodium hypochlorite (3 minutes), 70% ethanol (30 seconds), and 3% sodium thiosulfate, then rinsed three times with sterile distilled water. The sterilized tissues were cut into 5 mm pieces and placed on starch casein agar (SCA) supplemented with cycloheximide (50 µg/mL) and nalidixic acid (20 µg/mL) to inhibit fungal and non-actinomycete bacterial growth (11).

Cultures were incubated at 28 °C for up to four weeks, with regular monitoring for colony growth. Putative actinomycete colonies were purified by repeated streaking on ISP2 medium. To verify the effectiveness of the sterilization process, 100 µL of the final rinse solution was plated on SCA and incubated at 28 °C for two weeks.

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 earlier studies (12) followed by polymerase chain reaction (PCR) with taxon-specific primers (Table 1) to identify actinomycetes, as previously demonstrated (13).

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	(13)
ACT878r	CCGTACTCCCCAGGCGGGG			
A3F	GCSTACSYSATSTACACSTCSGG	*NRPS*	700-800	(14)
A7R	SASGTCVCCSGTSCGGTAS			
KIF	TSAAGTCSAACATCGGBCA	*PKS-I*	1200-1400	(15)
M6R	CGCAGGTTSCSGTACCAGTA			
PKS-II-A	TSGCSTGCTTCGAYGCSATC	*PKS-II*	600	(14)
PKS-II-B	TGGAANCCGCCGAABCCGCT			

2. 3 Evaluation of Antibacterial Activity of *Actinomycete*s

All actinomycetal isolates were fermented, and their resulting extracts were screened according to previous research without modifications (11).

The drug-sensitive and drug-resistant bacteria, as selective members of the ESKAPE 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 (approximately 1.5×10^8 CFU/mL).

Bacterial lawns were prepared on Mueller-Hinton (MH) agar with 6 mm wells, following the procedure outlined by Hajizadeh et al. (10). Into each well, 100 µL of crude extracts were added. The plates were left at room temperature for one hour before incubating at 37ºC for 24 hours. After

incubation, the inhibition zones were measured millimeters (mm) using 100 µL of ethyl acetate as a control.

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. Phenotypic Identification of *Actinomycetes*

Endophytic isolates exhibiting chalky, hard, and leathery colony morphologies on culture media, particularly those with distinct color variations between the upper and lower surfaces, were preliminarily identified as *Actinomycetes*. Based on these characteristics, 54 isolates, all recovered from olive tree roots, were classified as *Actinomycetes*.

3.2. Molecular Identification of *Actinomycetes*

PCR amplification of the 16S rRNA gene was performed on DNA extracted from the isolated endophytes. A positive PCR product was obtained for 45 out of 54 strains (83.3%). Consequently,

subsequent molecular analyses and phenotypic evaluations of antimicrobial properties were conducted on these 45 actinomycete isolates. Figure 1 illustrates the positive PCR amplification of the 16S rRNA gene for several endophytic isolates in this study.

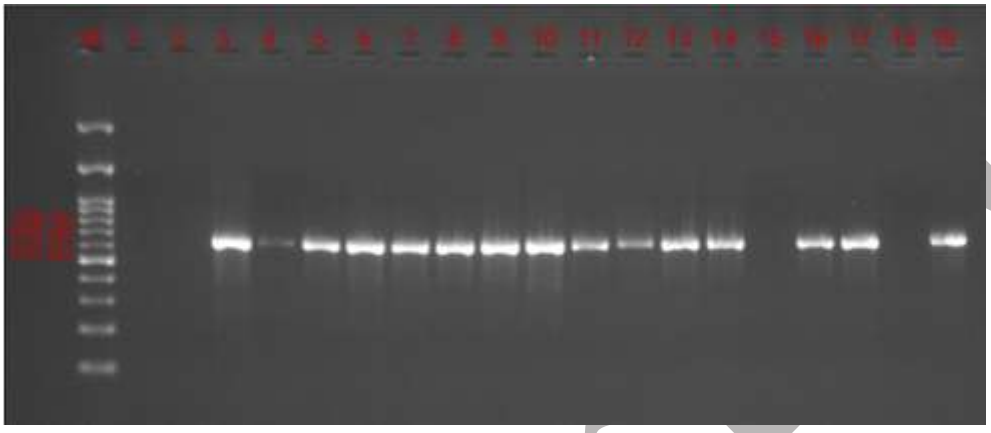

Figure 1: Results of PCR product electrophoresis for several endophytic isolates in this study.

- Lanes: M) DNA size marker, 1) Negative control, 2-19) PCR products of the isolates (640 base pairs)

3.3. Antimicrobial Activity of Isolated *Actinomycetes*

The antimicrobial activity of *Actinomycetes* isolated from olive trees was assessed by measuring the diameter of the clear zone surrounding the wells. Among the 45 isolates with a positive PCR result for the 16S rRNA gene, 16 isolates (35.6%) exhibited antimicrobial activity against the tested pathogenic bacteria. Of these 16 isolates, 12 (75%) were active against drug-sensitive *Staphylococcus aureus*, 11 (68.8%) against drug-sensitive *Pseudomonas aeruginosa*, 4 (25%) against drug-resistant *S. aureus*, 7 (43.8%) against drug-resistant *Klebsiella pneumoniae*, 2 (12.5%) against drug-resistant *P. aeruginosa*, and 5 (31.25%) against drug-sensitive *K.*

pneumoniae. None of the *Actinomycetes* in this study exhibited activity against *Acinetobacter baumannii*.

3.4. PCR Screening for *NRPS*, *PKS-I*, and *PKS-II* Genes

In addition to phenotypic evaluation of antimicrobial activity, the presence of *NRPS*, *PKS-I*, and *PKS-II* genes was investigated in the *Actinomycetes* with positive 16S rRNA gene PCR results. Of the 45 isolates, 30 (66.7%) possessed the *NRPS* gene with a length of 700-800 base pairs, 13 (28.9%) harbored the *PKS-I* gene with a length of 1200 base pairs, and 42 (93.3%) carried the *PKS-II* gene with a length of 600 base pairs (Figures 2-4).

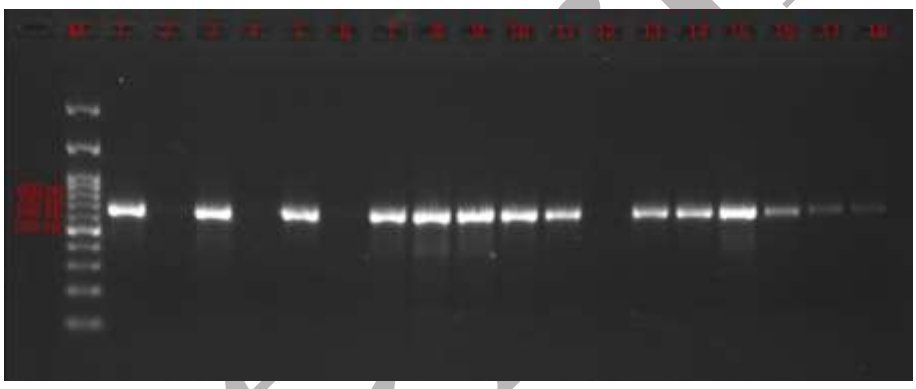

Figure 2. Agarose gel electrophoresis of *NRPS* gene PCR products from actinomycetal isolates.
Lanes: M) DNA size marker; 1) Positive control; 2) Negative control; 3–18) PCR products from actinomycete isolates, displaying bands between 700–800 bp, indicative of the amplified *NRPS* gene.

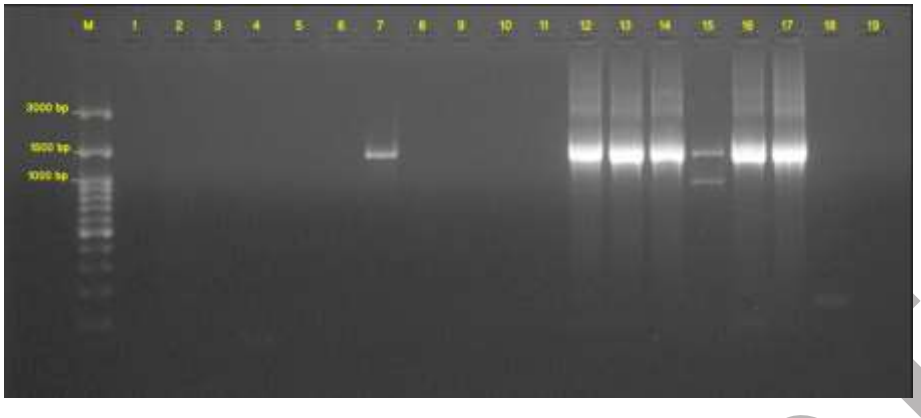

Figure 3. Agarose gel electrophoresis of *PKS-I* gene PCR products from actinomycetal isolates.
Lanes: M) DNA size marker; 1: Negative control; 2–19) PCR products from actinomycete isolates showing a band at approximately 1200-1400 bp, representing the amplified *PKS-I* gene.

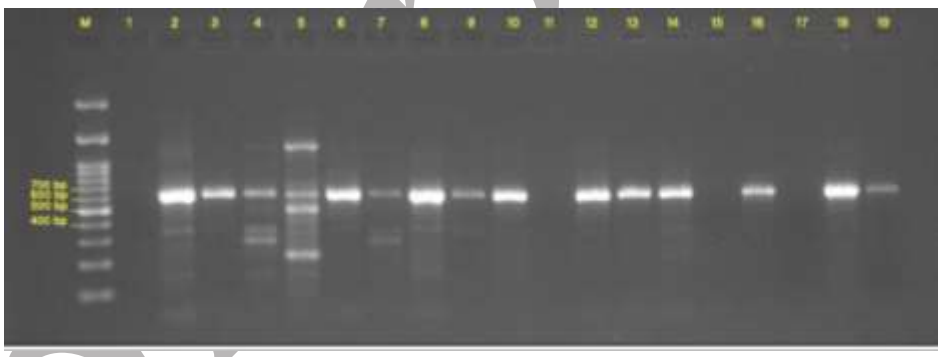

Figure 4. Agarose gel electrophoresis of *PKS-II* gene PCR products from actinomycetal isolates.
Lanes M) DNA size marker; 1) Negative control; 2–19) PCR products from actinomycete isolates exhibiting a band at 600 bp, corresponding to the amplified *PKS-II* gene.

3.5. Correlation between Antimicrobial Activity and presence of Biosynthetic Gene Clusters

The relationship between antimicrobial activity and BGCs is detailed in Table 3. Strains with identified BGCs often exhibit antibacterial activity, highlighting the role of these clusters in producing antimicrobial compounds. Strains possessing *PKS-II* and *NRPS* clusters are more likely to show activity against both drug-resistant and drug-sensitive pathogens.

Notably, strains Z10 and Z36, which contain all three types of clusters, demonstrated strong antibacterial effects against multiple pathogens. While antimicrobial activity was observed against drug-sensitive pathogens as well, the presence of BGCs in these cases was less consistent.

Table 3. Correlation between the Antibacterial Activity and the presence of BGCs

No	Code	*NRPS*	*PKS-I*	*PKS-II*	33591[a]	2774[b]	700603[c]	10031[d]	27853[e]	25923[f]	BAA-747
1	Z2	*		*							
2	Z4		*	*							
3	Z5	*		*			*		*	*	
4	Z6	*		*							
5	Z7	*		*							
6	Z8	*	*	*							
7	Z9	*		*						*	
8	Z10	*	*	*				*	*	*	
9	Z11				*		*	*	*	*	
10	Z12		*	*							
11	Z13	*		*						*	
12	Z15	*		*					*	*	
13	Z16								*		
14	Z17	*		*					*	*	
15	Z18	*							*	*	
16	Z19			*							
17	Z22	*		*							
18	Z23	*		*							
19	Z24	*		*							
20	Z25	*		*							
21	Z26	*		*							
22	Z27	*	*	*	*				*	*	
23	Z28	*		*							
24	Z29		*	*							
25	Z31			*		*					
26	Z32	*	*	*							
27	Z33	*		*							
28	Z34			*							
29	Z35		*	*							
30	Z36		*	*	*	*	*	*	*	*	
31	Z37	*		*							
32	Z38	*	*	*			*	*	*	*	
33	Z39		*	*							
34	Z41	*	*	*							
35	Z42			*							
36	Z43	*		*							
37	Z44	*		*							
38	Z45	*		*							
39	Z46	*		*							
40	Z48		*	*	*		*	*	*	*	
41	Z49			*			*				
42	Z50			*			*				
43	Z51	*		*							
44	Z52	*		*							
45	Z54	*		*							

a= *S. aureus* (ATCC 33591) (Drug resistant); b= *P. aeruginosa* (ATCC 2774) (Drug resistant); c= *K. pneumoniae* (ATCC 700603) (Drug resistant); d= *K. pneumoniae* (ATCC 10031) (Drug sensitive); e= *P. aeruginosa* (ATCC 27853) (Drug sensitive); f= *S. aureus* (ATCC 25923) (Drug sensitive); g= *A. baumanii* (ATCC BAA-747).

4. Discussion

The study of 54 bacterial isolates revealed that 45 (83.33%) were identified as *Actinomycetes*, all sourced from the root tissues of the plant. This finding aligns with existing literature that highlights the dominant role of *Actinomycetes* in plant root microbiomes, where they contribute to plant health and secondary metabolite production (17). Roots serve as primary sites for endophytic colonization due to their direct contact with soil, which acts as a reservoir for *Actinomycetes*. The function of roots as "gatekeepers" has been described, showing how they selectively screen soil bacteria, resulting in a microbiome that is predominantly composed of *Actinomycetota*, including *Actinomycetes* (8). This selective colonization is essential for plant growth and stress resistance.

The presence of biosynthetic genes, such as nonribosomal peptide synthetases (*NRPS*) and polyketide synthases (*PKS-I* and *PKS-II*), in a significant percentage of isolates, highlights the potential of *Actinomycetes* to produce diverse secondary metabolites. Specifically, frequencies of these genes were reported as 66.7% for *NRPS*, 28.9% for *PKS-I*, and 93.3% for *PKS-II* among isolates from *Olea europaea*. This suggests that *Actinomycetes* associated with this plant could be valuable sources for discovering novel bioactive compounds, particularly antimicrobial agents, as these gene clusters are often linked to the biosynthesis of such metabolites (17). However, the absence of a direct correlation between biosynthetic genes and antibacterial activity indicates that additional factors, such as gene expression, regulatory mechanisms, and environmental conditions, play crucial roles in the production of bioactive compounds. For instance, it has been noted that while many actinomycetes harbor biosynthetic gene clusters, not all express these genes under laboratory conditions, leading to variability in antimicrobial activity (18). The antibacterial activity of the isolates against drug-resistant pathogens is particularly significant given the global rise in antibiotic resistance, which poses a major public health challenge. Among the 45 isolates studied,

16 (35.6%) demonstrated antibacterial activity, with the highest efficacy observed against drug-sensitive *S. aureus* and *P. aeruginosa*. This finding is crucial as these bacteria are common causes of infections and are often resistant to multiple antibiotics (19). The identification of isolates with significant antibacterial properties against these pathogens suggests potential avenues for developing new therapeutic agents (20). Moreover, research into endophytic *Actinomycetes* from medicinal plants supports their antibacterial potential. It has been reported that 69% of isolates from medicinal plants in Iran exhibited antimicrobial activity against various pathogens, including *S. aureus* and *Escherichia coli* (21).

In this study, strains Z10 and Z36 were identified as particularly noteworthy due to their possession of all three types of BGCs and their pronounced antibacterial activity. These findings align with previous research demonstrating the antimicrobial potential of *Actinomycetes* isolates harboring multiple BGCs. For instance, *Streptomyces* sp. KN37, isolated from extreme environments, exhibited robust antimicrobial activity and contained 41 predicted BGCs, some of which resemble known antibiotic-producing gene clusters, underscoring its potential for novel antibacterial compound discovery (22). Similarly, *Actinomycetes* isolates from mangrove sediments revealed that 19 strains possessed BGCs, with three displaying significant antibacterial activity against pathogens such as *S. aureus* and *E. coli*, further supporting the correlation between BGC presence and antimicrobial efficacy (23).

Despite the presence of biosynthetic genes in some isolates, the absence of antibacterial activity suggests that these genes may be silent or require specific activation conditions. It was found that many *Actinomycetes* from mangrove sediments contained biosynthetic gene clusters but exhibited limited antibacterial activity, indicating that the presence of these genes does not guarantee expression under standard conditions (24).

The phenomenon of "cryptic biosynthesis" is prevalent in *Actinomycetes*, where many biosynthetic gene clusters remain silent under standard laboratory conditions. Advanced techniques such as genome mining and metabolic engineering are essential to unlock their full biosynthetic potential. Strategies such as co-cultivation, external cues, and genetic manipulation have been employed to activate these silent pathways, revealing previously uncharacterized compounds (24). Additionally, the identification of isolates exhibiting antibacterial activity without known biosynthetic genes suggests alternative mechanisms for antibiotic production. Research indicates that atypical response regulators and alternative sigma factors can modulate antibiotic biosynthesis through mechanisms not yet fully understood (25). This underscores the need for further investigation into these alternative pathways to enhance our understanding of antibiotic production and discover new therapeutic agents.

The isolation of *Actinomycetes* from *Olea europaea* is significant for drug discovery, particularly in addressing multidrug-resistant infections. Research shows that extracts from *Olea europaea* exhibit antibacterial activity against various resistant strains, including *Mycobacterium tuberculosis* (26). The presence of bioactive compounds such as oleuropein in olive leaves contributes to their antimicrobial properties, making them potential candidates for developing new antibiotics (27). Furthermore, the traditional use of *Olea europaea* in various cultures for treating infections highlights its therapeutic potential. The exploration of its *Actinomycetes* isolates could lead to the discovery of novel antimicrobial agents capable of combating the growing challenge of antibiotic resistance (28). Overall, this study enriches our understanding of plant-based antimicrobial properties and opens avenues for innovative drug development against resistant pathogens.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors express gratitude to the Vice Chancellor for Research and Technology at Ilam University, Ilam, Iran, for their partial financial support of this study.

Ethics

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

Data Availability

The data supporting the findings of this study are not publicly available due to not being necessary for the public. However, they can be made available upon request from the corresponding author.

Authors' Contributions

1- Study concept and design: P. N., and M. N.

2- Acquisition of data: P. N., and F. P.

3- Analysis and interpretation of data: P. N., F. P., and M. N.

4- Drafting of the manuscript: P. N. and F. P.

5- Critical revision of the manuscript for important intellectual content: F. P., and M. N.

6- Statistical analysis: P. N., and M. N.

7- Administrative, technical, and material support: F. P., and M. N.

8- Study supervision: F. P., and M. N.

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