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Research Paper

Isolation, Molecular Characterization, and Antibacterial Activity of Actinomycetes Associated With Horsemint (*Mentha longifolia*)

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How to cite this article Nemati S, Pourahmad F, Nemati M. Isolation, Molecular Characterization, and Antibacterial Activity of Actinomycetes Associated With Horsemint (*Mentha longifolia*). Archives of Razi Institute Journal. 2025; 80(6):1497-1506. https://doi.org/10.32598/ARI.80.6.3487

https://doi.org/10.32598/ARI.80.6.3487

Article info:
Received: 12 May 2025
Accepted: 19 Jun 2025
Published: 01 Nov 2025

Keywords:
Actinomycetes, Antibiotic properties, *Mentha longifolia*, Endophytes, Biosynthetic gene clusters (BGCs)

ABSTRACT

Introduction: Actinomycetes, renowned for their antibiotic-producing capabilities, represent a promising avenue for the discovery of novel antimicrobial agents, especially amid the global challenge of antibiotic resistance. This study explored the endophytic actinomycetes associated with horsemint (*Mentha longifolia*), a medicinal plant known for its antimicrobial properties, to uncover potential sources of novel antibiotics.

Materials & Methods: Actinomycetes were isolated from horsemint samples collected in Ilam Province, Iran. Forty isolates were identified based on morphological and molecular analyses, including *16S rRNA* gene amplification. Antibacterial activity was evaluated against clinically relevant pathogens, including ESKAPE bacteria. Six isolates exhibited significant inhibitory effects, particularly against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, with some also active against *Staphylococcus aureus*.

Results: Further analysis revealed the presence of biosynthetic gene clusters (BGCs), including non-ribosomal peptide synthetases (*NRPS*) and polyketide synthases (*PKS-I* and *PKS-II*), in several isolates. Notably, strains such as *T35* and *T37* harbored all three gene types and demonstrated broad-spectrum antibacterial activity. Strain *B22*, containing the *NRPS* gene, showed significant inhibition of both drug-resistant and drug-sensitive pathogens. However, some isolates with BGCs exhibited no antibacterial activity, suggesting that gene expression and metabolite production are influenced by regulatory or environmental factors.

Conclusion: This study highlights the untapped potential of *M. longifolia*-associated Actinomycetes as a source of bioactive compounds. The discovery of strains with robust antibacterial activity underscores their value in addressing the urgent need for new antimicrobial agents, especially in combating antibiotic resistance. These findings also emphasize the importance of plant-microbe interactions in natural product biosynthesis. Future work should focus on optimizing conditions for activating silent gene clusters and further characterizing the therapeutic potential of these bioactive compounds.

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1. Introduction

Endophytes are specialized microorganisms, including bacteria and fungi, that reside within the internal tissues of plants without causing any harm. They play a crucial role in promoting plant health by enhancing growth, improving resistance to diseases, and aiding in stress management [1]. These microorganisms interact with the plant's microbiome, influencing various physiological processes and contributing to nutrient acquisition and phytohormone modulation [2]. Recognized as beneficial symbionts, endophytes produce bioactive compounds that help plants combat biotic and abiotic stresses, such as drought and salinity [3]. Their ability to synthesize secondary metabolites has significant implications for agriculture and medicine, making them valuable for sustainable practices [4]. They enhance nutrient uptake, inhibit pathogen growth, and improve resilience to environmental stresses, thereby promoting overall plant health [3]. Furthermore, endophytes can induce systemic resistance in plants, activating defense-related genes and strengthening the plant's immune response to various stresses [5].

Among bacterial endophytes, actinomycetes are particularly notable for their ability to produce a wide range of bioactive compounds, including antibiotics, antitumor agents, and immunosuppressants. These compounds have significant applications in agriculture and medicine, providing natural alternatives to synthetic chemicals [6]. Actinomycetes contribute to plant health by producing metabolites that protect against pathogens and enhance stress tolerance, making them invaluable for sustainable agricultural practices [7]. Actinomycetes are gram-positive, filamentous bacteria characterized by their high guanine-cytosine (G+C) content, which typically exceeds 55 mol% and can reach up to 75% [8]. These bacteria are distinguished by their unique morphological features, including branching hyphae [9]. They play a significant role in the production of bioactive compounds, contributing to the development of antibiotics, antitumor agents, and other secondary metabolites crucial for both medical and agricultural applications [8]. The genus *Streptomyces*, in particular, is responsible for producing more than two-thirds of clinically useful antibiotics, highlighting its ecological role in soil environments, where it competes with other microorganisms. This competition drives the evolution of new antibiotic compounds, making *Streptomyces* a vital resource for drug discovery and development [10].

The rise of antimicrobial resistance (AMR) has discovered new antibiotics a critical global priority. Natural habitats, especially those associated with medicinal plants, are considered valuable reservoirs of microbial diversity and bioactive compounds. Plant microbiomes, including endophytic communities, are shaped by ecological and evolutionary pressures, leading to the production of unique metabolites with potential therapeutic applications [11]. Understanding these dynamics can facilitate the harnessing of plant microbiomes for developing new therapeutic agents.

Mentha longifolia, commonly known as horsemint, is a medicinal plant belonging to the Lamiaceae family. This perennial herb is recognized for its traditional uses in various cultures, particularly for treating ailments such as sore throats and mouth irritations [12]. *M. longifolia* has been traditionally employed in folk medicine for its antibacterial, antifungal, and antioxidant properties. The plant is rich in essential oils, such as thymol and carvacrol, which are known for their therapeutic effects. These properties make thymol and carvacrol valuable in both therapeutic and commercial contexts [13].

Despite its extensive use in traditional medicine, the microbial community associated with *M. longifolia*, particularly its actinomycetes, has not been extensively studied. While existing research has explored the antimicrobial properties of *M. longifolia* extracts against various pathogens, there is a notable gap in studies focusing specifically on its associated microbial communities, such as actinomycetes. This study aimed to bridge this gap by isolating and characterizing actinomycetes from *M. longifolia* and investigating their potential to produce antibiotics. Additionally, the presence of biosynthetic gene clusters, such as non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), was examined to establish the genetic basis of their bioactive potential.

2. Materials and Methods

2.1. Sample collection and preparation

Samples of horsemint (roots, stems, leaves) were collected from various regions in Ilam Province, Iran, during the fall and winter of 2023. Plant tissues were surface-sterilized to remove external contaminants, and sections were aseptically prepared for microbial isolation.

2.2. Isolation and cultivation of actinomycetes

Tissue samples were plated on starch casein agar supplemented with cycloheximide (50 µg/mL) and potas-

Table 1. List of oligonucleotide primers used in the study

Ref.	Product Size (bp)	Annealing Temperature (°C)	Sequence (5'-3')	Target Gene
[15]	640	65	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	*16S rRNA*
[16]	700-800	58	GCSTACSYSATSTACACSTCSGG SASGTCVCCSGTSCGGTAS	*NRPS*
[17]	1200-1400	60	TSAAGTCSAACATCGGBCA CGCAGGTTSCSGTACCAGTA	*PKS-I*
[16]	600	60	TSGCSTGCTTCGAYGCSATC TGGAANCCGCCGAABCCGCT	*PKS-II*

sium dichromate (25 µg/mL) to inhibit fungal growth. The plates were incubated at 28 °C for 7–30 days. Colonies exhibiting characteristic chalky, dry morphologies were then subcultured onto ISP2 medium for purification.

2.3. DNA extraction and molecular characterization of actinomycetes

Genomic DNA was extracted from endophytic isolates using a boiling method [14], and the DNA concentration and purity were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The isolates were identified through PCR amplification using Actinomycetes-specific primers (Table 1), following the protocol described in the previous research [15].

2.4. Evaluation of antibacterial activity of actinomycete

All actinomycetal isolates were fermented, and their resulting extracts were screened according to previous research without modifications [14].

The antibacterial activity of the actinomycetal strains was evaluated using reference strains of ESKAPE pathogens (Table 2). The term ESKAPE pathogens refers to a group of clinically significant microorganisms, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., known for their ability to 'escape' the effects of conventional antibiotics due to their high levels of AMR. The bacteria were cultured overnight at 37 °C in Mueller-Hinton (MH) broth,

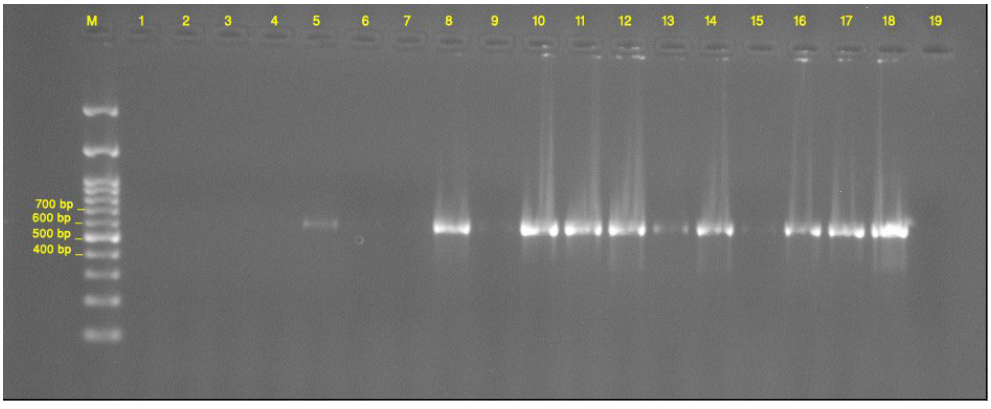

Figure 1. Agarose gel (1.2%) electrophoresis of *16S rRNA* PCR products from bacterial isolates

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

Table 2. Members of ESKAPE pathogens included in the 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	-

and the culture was then adjusted to a turbidity equivalent to 0.5 McFarland standards.

Bacterial lawns were created on MH agar with 6 mm wells, into which 100 µL of crude extracts were added [14]. The plates were left at room temperature for one hour before incubating at 37 ˚C. After 24 hours, inhibition zones were assessed in millimeters (mm), with 100 µL of ethyl acetate serving as the control.

2.5. Detection of biosynthetic gene cluster (BGC) genes

Using the specific primers listed in Table 1, the presence of *PKS-I*, *PKS-II*, and *NRPS* genes was investigated by PCR following previously described protocols [16, 17].

3. Results

3.1. Phenotypic identification of isolates

The endophytic isolates exhibited chalky, hard, and leathery colony textures with diverse pigmentation, notably displaying distinct coloration between the upper and lower colony sections. Based on these characteristics, the isolates were preliminarily identified as Actinomycetes. In total, 54 isolates were categorized within this group.

3.2. Molecular identification of actinomycete

Polymerase chain reaction was conducted on DNA extracted from the isolated bacteria, successfully amplifying the *16S rRNA* gene in 40 out of 54(74.1%) endophytic isolates. The PCR products measured approximately 640 base pairs, confirming the affiliation of these isolates with the actinomycetes class (Figure 1). The identification of isolates revealed that 10(59%) originated from the root, 18(82%) from the stem, and 12(80%) from horsemint leaves.

3.3. Antibacterial activity of actinomycetal isolates

Of the 40 molecularly confirmed isolates, 6(15%) demonstrated antibiotic activity against the tested pathogenic bacteria (Figure 2). All six isolates (100%) displayed antibacterial activity against drug-sensitive *P. aeruginosa* and *K. pneumoniae*. Five isolates (83.3%) were active against both drug-resistant and drug-sensitive *S. aureus*. However, none of the isolates showed activity against drug-resistant *A. baumannii* or *P. aeruginosa*.

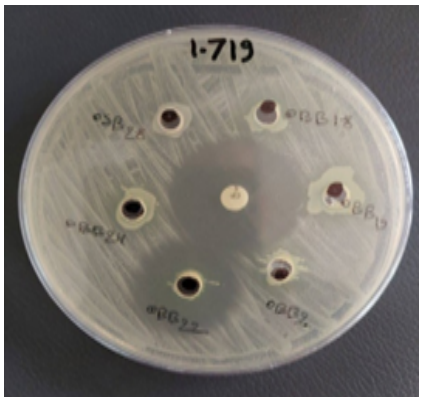

Figure 2. A representative selection of antibacterial activity of the isolates against *K. pneumoniae* (ATCC 700603)

Note: The ciprofloxacin disk was used as a positive control in the center of the MH medium.

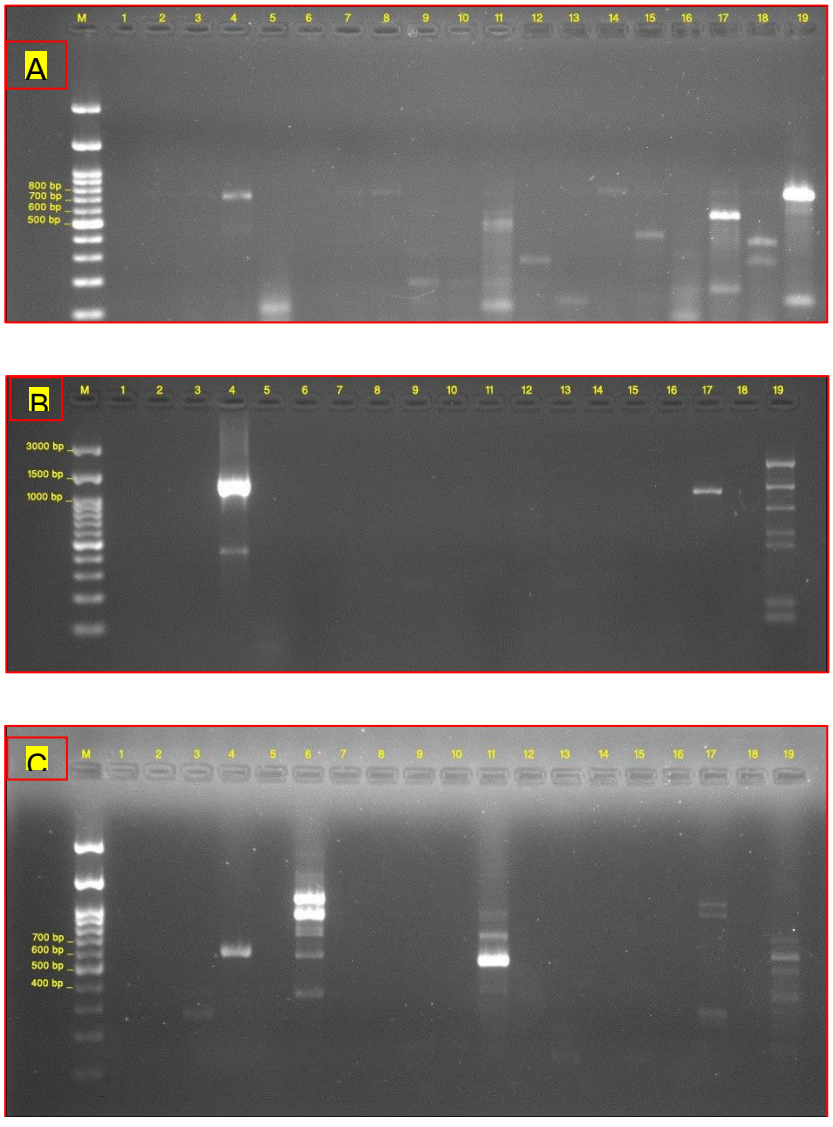

Figure 3. Agarose gel (1.2%) electrophoresis of PCR products from actinomycete isolates

A) *NRPS* gene PCR products: Lane 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.

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

C) *PKS-II* gene PCR products: Lane 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.

3.4. Detection of BGCs

Among the 40 isolates with positive *16S rRNA* gene PCR results, 28 isolates (70%) contained the *NRPS* gene (700-800 base pairs), 8 isolates (20%) contained the *PKS-I* gene (1200 base pairs), and 22 isolates (55%) contained the *PKS-II* gene (600 base pairs) (Figure 3).

3.5. Correlation between antimicrobial activity and presence of BGCs

The connection between antimicrobial activity and BGCs is outlined in Table 3. Among the six isolates exhibiting inhibitory activity against reference pathogenic bacteria, strain B22 was identified to harbor the *NRPS* gene and showed inhibitory effects against four bacterial strains, including both drug-resistant and drug-sensitive *S. aureus* and *K. pneumoniae*. Strains T35 and T37 pos-

Table 3. Correlation between the antibacterial activity and the presence of BGCs

No.	Isolate Code	BGCs			ESKAPE Pathogens						
		NRPS	*PKS-I*	*PKS-II*	33591[a]	2774[b]	700603[c]	10031[d]	27853[e]	25923[f]	BAA-747[g]
1	OS3	*									
2	OS6	*									
3	OS8	*	*	*							
4	OS9										
5	OS10/1			*							
6	OS10/2	*									
7	OS11	*									
8	OS13/1										
9	OS13/2										
10	OS14	*		*							
11	B16										
12	B17										
13	B18	*									
14	B21	*									
15	B22	*			*		*	*		*	
16	B24	*	*								
17	B25										
18	B26	*	*	*							
19	B27	*		*							
20	T28	*		*							
21	T29	*									
22	T30	*	*	*							
23	T32	*									
24	T33	*	*								
25	T34	*		*							
26	T35	*	*	*	*		*	*	*	*	
27	T37	*	*	*	*		*	*	*	*	
28	T38	*			*		*	*	*	*	
29	T39	*		*							
30	T41	*		*	*		*	*	*	*	

No.	Isolate Code	BGCs			ESKAPE Pathogens						
		NRPS	*PKS-I*	*PKS-II*	33591[a]	2774[b]	700603[c]	10031[d]	27853[e]	25923[f]	BAA-747[g]
31	T42	*		*							
32	P43			*	*		*	*	*		
33	P44			*							
34	P45			*							
35	P47	*		*							
36	P48	*	*	*							
37	P49	*		*							
38	M50	*		*							
39	M51			*							
40	M52			*							

[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).

sessed all three biosynthetic genes and inhibited five bacteria strains, including both drug-resistant and drug-sensitive *S. aureus*, *K. pneumoniae*, and drug-sensitive *P. aeruginosa*. Strain T38 also carried the *NRPS* gene and exhibited the same inhibitory profile identical to that T35 and T37. Strain T41 contained both the *NRPS* and PKS-II genes and inhibited all five bacterial strains: Drug-resistant and drug-sensitive *S. aureus*, *K. pneumoniae*, and drug-sensitive *P. aeruginosa*. Additionally, strain P43, which possessed the *PKS-II* gene, inhibited three gram-negative bacteria: Drug-resistant and drug-sensitive *K. pneumoniae* and drug-sensitive *P. aeruginosa*.

Conversely, several isolates containing biosynthetic gene clusters demonstrated no antibacterial activity. Strains OS14, B24, B27, T28, T33, T34, T39, T42, P47, P49, and M50 carried both *NRPS* and *PKS-II* genes but showed no inhibitory effects. Similarly, strains OS8, B26, T30, and T48 possessed all three biosynthetic genes yet exhibited no activity against the tested bacteria.

4. Discussion

The present study aimed to isolate and characterize the endophytic microbiota associated with horsemint (*M. longifolia*) and assess their antibacterial activities against a range of pathogenic bacteria. The findings reveal a complex relationship between the presence of biosynthetic gene clusters and the observed antibacterial activity, highlighting both the potential and the limitations of harnessing microbial metabolites for therapeutic applications. Among the isolates, strain B22 harbored the *NRPS* gene and exhibited significant inhibitory effects against both drug-sensitive and drug-resistant strains of *S. aureus* and *K. pneumoniae*. This finding aligns with previous studies demonstrating a correlation between *NRPS* genes and the production of bioactive compounds with antibacterial properties [18]. The ability of B22 to inhibit clinically relevant pathogens underscores the potential of horsemint-associated microbes as promising sources of novel antimicrobial agents, particularly in the face of rising antibiotic resistance.

Strains T35 and T37 further exemplified the potential of endophytes from horsemint, as they not only possessed all three biosynthetic gene clusters but also demonstrated inhibitory activity against a broader spectrum of bacteria including drug-sensitive *P. aeruginosa*. Multiple biosynthetic pathways suggest a robust capacity for metabolite production, consistent with previous findings that indicated a greater diversity in antibacterial activity correlates with the complexity of the biosynthetic machinery in microbial isolates [19].

Interestingly, strain T38, which also carried the *NRPS* gene, exhibited an inhibitory profile identical to that of T35 and T37, indicating that the presence of specific biosynthetic genes may lead to redundant metabolic pathways capable of producing similar antimicrobial compounds [20]. Conversely, despite the presence of both

NRPS and *PKS-II* genes, the lack of antibacterial activity in several isolates raises critical questions regarding the expression of these biosynthetic pathways. This phenomenon has been documented in other studies, where the mere presence of biosynthetic gene clusters did not guarantee the production of bioactive metabolites [21]. It is plausible that environmental factors, growth conditions, or regulatory mechanisms within the microbial community may influence gene expression and metabolite production, warranting further investigation into the conditions that may activate these silent pathways.

While the delineation of species was not conducted within the context of this investigation at the genus and species levels, it is frequently observed that the genus *Streptomyces* emerges as the predominant entity within endophytic communities [22], suggesting that various plant tissues afford distinct ecological niches.

In medicine, the bioactive compounds of actinomycetes present a promising avenue for new therapeutic agents. Metabolomics integrates various omics data to identify and characterize genes involved in natural product biosynthesis, enhancing the discovery of novel compounds and their therapeutic potentials. Genomic editing, particularly using CRISPR/Cas9, allows for precise modifications in organisms like yeast, facilitating the reconstruction of complex metabolic pathways [23]. In vivo studies are essential for validating the therapeutic efficacy and safety of new treatments.

For instance, research on 5-aminolevulinic acid (5-ALA) in glioblastoma showed that it enhances radiotherapy without increasing toxicity [24]. Additionally, in vivo evaluations of new antimicrobial peptides, like dermaseptin-AC, demonstrated their effectiveness against resistant bacteria while assessing safety [25]. Such studies are critical for ensuring that new therapies are both effective and safe for clinical use.

This research highlights the untapped potential of *M. longifolia* as a reservoir of antibiotic-producing actinomycetes. Previous studies have demonstrated that extracts from this plant exhibit strong antimicrobial activity against various bacterial strains, including both gram-positive and gram-negative bacteria. For instance, extracts showed effectiveness against *S. aureus* and *K. pneumoniae*, underscoring their broad-spectrum antimicrobial properties [22]. Additionally, essential oils derived from *M. longifolia* have been reported to possess notable antimicrobial properties, making them promising candidates for further exploration in drug development [12]. The presence of bioactive compounds in *M. longifolia* contributes to its potential as a natural source for developing new antibiotics, particularly in the context of rising antibiotic resistance [11]. Thus, this research underscores the untapped potential of *M. longifolia* in the field of antimicrobial drug discovery.

5. Conclusion

The presence of *NRPS*, *PKS-I*, and *PKS-II* genes in the isolates confirms their ability to synthesize diverse bioactive compounds. These findings pave the way for future biotechnological applications aimed at addressing the urgent need for novel antimicrobial agents in the fight against antibiotic resistance. By leveraging the natural diversity of plant-associated microbes, this study contributes to the development of sustainable and innovative approaches to drug discovery.

In summary, this investigation elucidates that *M. longifolia* constitutes a significant resource for the isolation of Actinomycetes with antibiotic characteristics. The findings support the traditional utilization of this botanical species in medicinal practices and delineate promising avenues for discovering novel antimicrobial agents through comprehensive analysis of its endophytic microbiota.

Ethical Considerations

Compliance with ethical guidelines

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

Data availability

The data supporting the findings of this study are not publicly available,as public access isn't deemed necessary. However, they can be made available upon reasonable request from the corresponding author.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization, study design, and statistical analyses: Samaneh Nemati and Fazel Pourahmad; Samples collections, analysis, data interpretation, and writing the original draft: All Authors; Review and editing: Fazel Pourahmad and Mostafa Nemati.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

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.

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