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

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Abstract

Actinomycetes, renowned for their antibiotic-producing capabilities, represent a promising avenue in the search for novel antimicrobial agents, especially amidst 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. *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*.

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 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.

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.

Keywords: *Actinomycetes*, antibiotic properties, *Mentha longifolia*, endophytes, biosynthetic gene clusters

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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 enhancing 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 range 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

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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 made 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

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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*), were 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 potassium dichromate (25 µg/mL) to inhibit fungal growth. Plates were incubated at 28°C for 7–30 days. Colonies exhibiting characteristic chalky, dry morphologies were 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 via PCR amplification using Actinomycetes-specific primers (Table 1) as described in the previous research (15).

Table 1. List of oligonucleotide primers used in the study

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Target gene	Sequence (5'-3')	Annealing temperature (°C)	Product size (bp)	Reference
16S rRNA	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	65	640	(15)
NRPS	GCSTACSYSATSTACACSTCSGG SASGTCVCCSGTSCGGTAS	58	700-800	(16)
PKS-I	TSAAGTCSAACATCGGBCA CGCAGGTTSCSGTACCAGTA	60	1200-1400	(17)
PKS-II	TSGCSTGCTTCGAYGCSATC TGGAANCCGCCGAABCCGCT	60	600	(16)

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 available 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 antimicrobial resistance. The bacteria were cultured overnight at 37ºC in Mueller-Hinton (MH) broth, and the culture was then adjusted to a turbidity level of 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 37ºC.

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After 24 hours, the inhibition zones were assessed in millimeters (mm), utilizing 100 µL of ethyl acetate as the 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	-

2.5 Detection of Biosynthetic Gene Cluster (BGC) genes

Using specific primers listed in Table 1, the presence of *PKS-I*, *PKS-II,* and *NRPS* genes was investigated by PCR as described elsewhere (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 under 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

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the *Actinomycetes* class (Figure 1). The identification of isolates revealed that 10 (59%) were from the root, 18 (82%) from the stem, and 12 (80%) from horsemint leaves.

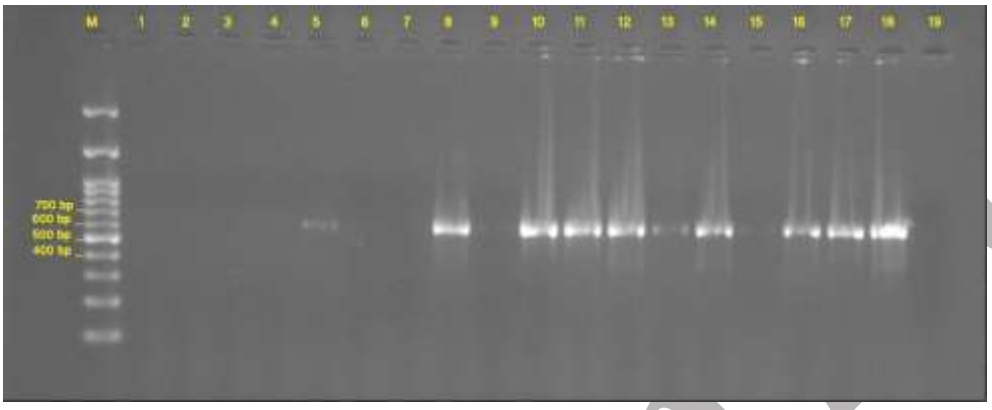

Figure 1. Agarose gel (1.2%) electrophoresis of 16S rRNA PCR products from bacterial isolates.
Lanes: M) DNA size marker; 1–19) PCR products from bacterial isolates showing a band at 640 bp, representing the amplified 16S rRNA gene.

3.3 Antibacterial Activity of 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 *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* Five isolates (83.3%) were active against both drug-resistant and drug-sensitive *Staphylococcus aureus*. However, none of the isolates showed any activity against drug-resistant *Acinetobacter baumannii* or *Pseudomonas aeruginosa*.

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

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

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).

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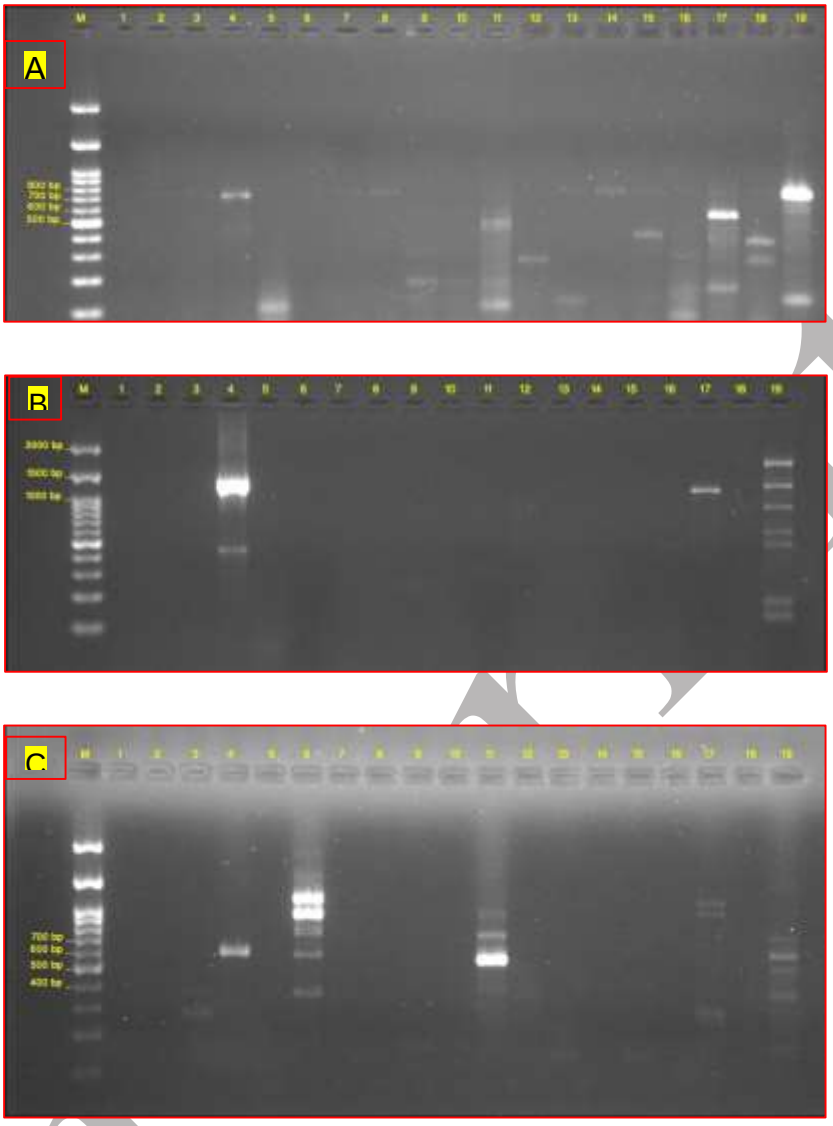

Figure 3. Agarose gel (1.2%) electrophoresis of PCR products from actinomycete isolates.

(A) *NRPS* gene PCR products. 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.

(B) *PKS-I* gene PCR products. 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.

(C) *PKS-II* gene PCR products. 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.

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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 possessed all three biosynthetic genes. These strains inhibited five bacteria, including 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 as T35 and T37. Strain T41 contained 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 with 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.

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

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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	*		*	*		*	*	*	*	
31	T42	*		*							
32	P43			*	*		*	*	*		
33	P44			*							
34	P45			*							
35	P47	*		*							
36	P48	*	*	*							
37	P49	*		*							
38	M50	*		*							
39	M51			*							
40	M52			*							

٢٣٦ a= *S. aureus* (ATCC 33591) (Drug resistant); b= *P. aeruginosa* (ATCC 2774) (Drug resistant); c= *K.*

٢٣٧ *pneumoniae* (ATCC 700603) (Drug resistant); d= *K. pneumoniae* (ATCC 10031) (Drug sensitive); e= *P.*

٢٣٨ *aeruginosa* (ATCC 27853) (Drug sensitive); f= *S. aureus* (ATCC 25923) (Drug sensitive); g= *A. baumanii*

٢٣٩ (ATCC BAA-747).

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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 limitations of harnessing microbial metabolites for therapeutic applications.

Among the isolates, strain B22 was identified to harbor the *NRPS* gene, and exhibited significant inhibitory effects against drug-sensitive and drug-resistant strains of *S. aureus* and *K. pneumoniae*. This finding aligns with previous studies that have established a correlation between the presence of *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 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 that produce similar antimicrobial compounds (20). Conversely, despite the presence of both *NRPS* and *PKS-II* genes, the lack of antibacterial activity

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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 source of antibiotic-producing *Actinomycetes*. Studies have demonstrated that extracts from this plant exhibit strong antimicrobial

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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, the essential oils derived from *M. longifolia* have been reported to possess notable antimicrobial activities, 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.

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, 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 sets the stage for sustainable and innovative approaches to drug discovery.

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

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

Authors' contributions

S. N. and F. P. proposed and designed the research, S.N., F. P., and M. N. collected samples

S. N., F. P., and M. N. analyzed and interpreted data, M. H., F. P., and M. N. drafted the manuscript, S. H. and F. P. performed statistical analyses, F. P. and M. N. proved the final version of the manuscript.

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