Original Article

Isolation and Screening of Antibacterial Activity of *Actinomycetota* from the Medicinal Plant, *Anthemis pseudocotula* Boiss

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ABSTRACT

Antibiotic resistance is rising dramatically worldwide, and thus the production of new antibiotics is indispensable. Recent scientific initiatives have focused on the bioprospecting of microorganisms' secondary metabolites, with a particular focus on the look for natural products with antimicrobial properties derived from endophytes. All plant species, regardless of their type, are thought to anchor endophytic bacteria (EB). There are many potential uses for the natural therapeutic compounds made by EB in medicine, agriculture, and the pharmaceutical industry. To investigate antibacterial properties in this study, *Actinomycetota* (formerly, *Actinobacteria*) were isolated from *Anthemis pseudocotula* Boiss., identified, and underwent bioprospecting by morphological and molecular methods. Samples were collected from Ilam, Iran, and then divided into roots, leaves, stems, and flowers. After disinfection, they were cut into 2 mm pieces, cultured on casein agar culture medium, and incubated at 28ºC for up to four weeks. *Actinomycetota* was identified using the polymerase chain reaction method targeting the 16S rRNA gene. To evaluate the antibacterial properties of the isolated *Actinomycetota*, the agar diffusion method was used. In parallel, the frequencies of biosynthetic gene clusters, including polyketide synthase (*PKS-I* and *PKS-II*) and nonribosomal peptide synthetase (*NRPS*) genes, were determined in the isolated *Actinomycetota*. Ninety bacteria were isolated from different parts of *Anthemis* flowers. Thirty-eight (42.2%) of these bacteria belonged to the phylum *Actinomycetota*, and out of these 38, 15 isolates (39.5%) had antibacterial properties. Of these, 11 isolates (73.3%) exhibited antibacterial effects against *Staphylococcus aureus*, 2 (13.3%) against *Pseudomonas aeruginosa*, 3 (20%) against *Escherichia coli*, and two isolates (13.3%) against *Salmonella enterica* sub-species of *enterica* serovar Typhimurium. The results of the molecular analysis of *PKS-I*, *PKS-II*, and *NRPS* genes showed that out of 38 isolated *Actinomycetota* strains, 23 isolates (60.5%) carried *PKS-I* gene, 6 (15.8%) harbored *PKS-II* gene, and 20 isolates (52.6%) had *NRPS* gene. This study indicates that *Anthemis pseudocotula* Boiss. has a number of active *Actinomycetota* that produce secondary metabolites with antibacterial properties.

Keywords: *Actinomycetota*, *Anthemis pseudocotula* Boiss., Antibacterial activity, Bioprospecting, Isolation

1. Introduction

Due to the dispersion of drug resistance among microbial pathogens, discovering novel antimicrobials is desirable. *Actinomycetota* (formerly, *Actinobacteria*), the biggest member of the domain bacteria, are distinguished producers of a wide variety of secondary metabolites, including about 75% of the total antibiotic production available worldwide (1, 2). The vast majority of antibiotics have been produced by the genus *Streptomyces*, which belongs to the phylum *Actinomycetota* by searching soil (3, 4). As a result, the discovery of known molecules remains a challenge while hunting *Actinomycetota* for antimicrobial compounds, which may have reduced the likelihood of discovering new biologically active molecules from them (5, 6). Therefore, it is important to look into *Actinomycetota*'s sourcing in novel niche habitats to find novel bioactive compounds. The plant endosphere is a sophisticated micro-ecosystem that allows for the habitation of a variety of niches by different microorganisms collectively known as endophytes. Endophytes are microorganisms that live inside the tissues of plants without harming the host plant or the surrounding environment (7). Endophytic microbes exploit unusual habitats, which might allow them to make bioactive substances similar to those produced by their host. As such, it is known that the *Actinomycetota* of medicinal plants can produce a variety of secondary metabolites (8).

In recent years, numerous reports have shown the metabolic potential of endophytic *Actinomycetota* (9), which has led to the discovery of numerous bioactive compounds (4, 10). A different approach would be to characterize *Actinomycetota* based on their metabolic potential by targeting numerous genes encoding for the production of bioactive compounds (11). Biosynthetic gene clusters (BGCs) of the secondary metabolites of Actinomycetotal strains are primarily encoded by polyketide synthases (*PKS-I* and *II*), as well as non-ribosomal peptide synthetase (*NRPS*) pathways (12, 13). The presence of these genes, therefore, points to the high potential of *Actinomycetota* for producing biologically active compounds.

Since the Roman times, members of the genus *Anthemis*, commonly known as chamomile (Family: *Asteraceae*), have been used in traditional medicine with numerous therapeutic applications due to their antioxidant, antimicrobial, antispasmodic, and anti-inflammatory qualities. Despite the widespread use of this plant in ethnobotanical medicine, there have been few attempts to examine its chemical or biological characteristics regarding its potential for therapeutic use (14). Moreover, to our knowledge, the endosymbiotic bacteria associated with *Anthemis* species (spp.) have not been reported yet. Therefore, this study aimed to isolate endophytic *Actinomycetota* associated with *Anthemis pseudocotula* Boiss. and screen their antibacterial activity against test bacterial pathogens. Additionally, it focused on the detection of secondary metabolite genes that might be responsible for antibacterial activities.

2. Materials and Methods

2.1. Sample Collection and Isolation of Endophytic *Actinomycetota*

Anthemis pseudocotula Boiss., a healthy medicinal plant, was collected from the Ilam province in the southwest of Iran. After being transported to the lab in zipped plastic bags, the plant samples were subjected to a six-step surface sterilization process within 24 h with modification (15). The sterilized plant parts (roots, stems, leaves, and flowers) were aseptically fragmented into 2 mm parts and were spread over the starch casein agar (SCA) (16), supplemented with cycloheximide (50 µg/mL) and nalidixic acid (20 µg/mL) to conquer the growth of fungi and non-*Actinomycetota* bacteria, respectively. The culture media were incubated at 28ºC for up to four weeks with regular observations for the potential growth of new colonies. The putative Actinomycetotal colonies were purified by repeated streaking using the International *Streptomyces* Project 2 (ISP2) medium. One hundred µL of the final rinse solution was applied on SCA plates and incubated at

28ºC for two weeks to check microbial growth and the results of surface sterilization.

2.2. DNA Isolation and Molecular Identification of *Actinomycetota*

Genomic DNA extractions were performed for all endophytic isolates using a simple boiling method, as described elsewhere (17), followed by polymerase chain reaction (PCR) with taxon-specific primers (Table 1) to identify *Actinomycetota*, as demonstrated previously (18).

2.3. Evaluation of Antibacterial Activity of *Actinomycetota*

Each Actinomycetotal isolate was cultured in trypticase soy broth (TSB) and ISP2 media at 28°C and shaken at 180 rpm. After 7 and 13 days of cultivation, the fermentation broth was centrifuged at 13000×g for 15 min for biomass removal. An equal volume of ethyl acetate was added to the supernatant and vigorously shaken. A vacuum rotary evaporator operating at 40°C was then used to evaporate the organic layer. The organic extracts were then used for antimicrobial activity screening. *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), *Salmonella enterica* sub-species (subsp.) *enterica* (*S. enterica*, ATCC 14028) and *Escherichia coli* (*E. coli*, ATCC 25922) were used to measure the antibacterial activity of Actinomycetotal strains. They were grown at 37ºC overnight in Mueller-Hinton (MH) broth, which had been adjusted subsequently to 0.5 McFarland standard turbidity. The pathogenic bacterial lawns were prepared on the MH agar whose wells of about 6 mm in diameter were made using a sterilized cork borer, and 50 µL of the crude extracts were added to each well. The added extract plates were left at room temperature for one hour to let the crude extract diffuse and then incubated at 37ºC. After 24 h, the diameters of the inhibition zones were measured in mm(s). The control was a 50 µL volume of ethyl acetate.

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

The genes encoding non-ribosomal peptide synthetase (*NRPS*) and polyketide synthases I and II (*PKS-I* and *II*) were amplified using three pairs of degenerate primers (Table 1). The PCR amplification was carried out as recommended elsewhere with minor modifications (19, 20). Briefly, PCR amplifications were performed based on the following thermal profiles: 5 min denaturation at 96ºC and 30 cycles of 1 min at 96ºC, 1 min at either 58ºC (for K1FM6R, A3F⁄A7R) or 60ºC (for KSα⁄KSß) and 1 min extension at 72ºC, followed by 7 min at 72ºC. Amplification products were checked by electrophoresis in 1.2% agarose gels.

Table 1. PCR primers used in this study

Primer name	Sequence (5'-3')	Target gene	Product size (bp)	Reference
Act 235F	CGCGGCCTATCAGCTTGTTG	16S rRNA	640	18
Act 878R	CCGTACTCCCCAGGCGGGG			
K1F	TSAAGTCSAACATCGGBCA	*PKS-I*	1200-1400	19
M6R	CGCAGGTTSCSGTACCAGTA			
KSα	TSGCSTGCTTGGAYGCSATC	*PKS-II*	600	20
KSß	TGG AANCCG CCGAABCCTCT			
A3F	GCSTACSYSATSTACACSTCSGG	*NRPS*	700-800	19
A7R	SASGTCVCCSGTSCGGTAS-			

3. Results

3.1. Isolation of Endophytic *Actinomycetota* and Non-*Actinomycetota*

In total, 90 endophytic bacterial isolates were obtained from the root, stem, leaf, and flowers of

Anthemis pseudocotula Boiss. Based on the results of 16S rRNA gene amplification using a set of phylum-specific primers, 38 isolates (42.2%) were identified as *Actinomycetota*. Out of 38 Actinomycetotal strains, the maximum number of isolates was obtained from the root (n=12, 31.6%), followed by flower (n=11, 28.9%), stem (n=8, 21.1%), and leaf (n=8, 21.1%) (Figure 1).

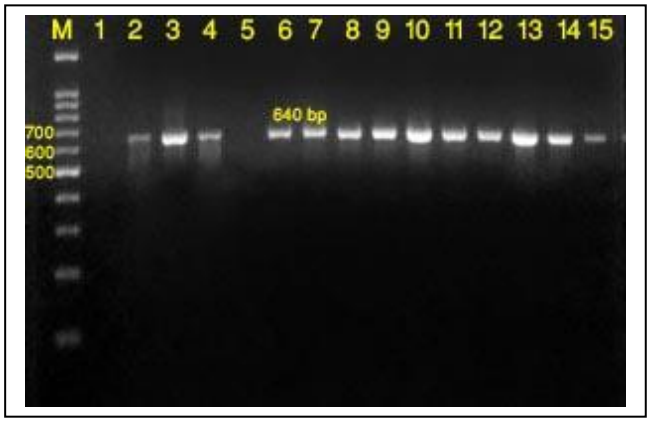

Figure 1. A representative selection of 16S rRNA gene PCR products obtained for actinomycetotal isolates
M= 100 bp DNA size marker
Lanes: 1) –ve control; 2) Isolate No 36; 3) Isolate No 37; 4) Isolate No 38; 5) Isolate No 39; 6) Isolate No 1; 7) Isolate No 2; 8) Isolate No 3; 9) Isolate No 4; 10) Isolate No 5; 11) Isolate No 6; 12) Isolate No 7; 13) Isolate No 8; 14) Isolate No 9; 15) Isolate No 10

3.2 Evaluation of Antimicrobial Activity of *Actinomycetota*

Out of 38 Actinomycetotal isolates, 15 isolates (39.5%) showed positive activity against test pathogens. Eleven isolates (73.3%) showed significant antimicrobial activity against *S. aureus*, while 3 (20%) were active towards *E. coli*. The reference strains of *P. aeruginosa* and *S. enterica* subsp. *enterica* serovar Typhimurium were inhibited by two *Actinomycetota* isolates (13.3%). Some isolates appeared to have a broad-spectrum antibacterial activity (inhibited three pathogenic microorganisms). For instance, isolate No. 2 suppressed the growth of *S. aureus*, *E. coli*, and *S. enterica* subsp. *enterica* serovar Typhimurium, and isolate No. 18 was active against *S. aureus*, *P. aeruginosa*, and *S. enterica* subsp. *enterica* serovar Typhimurium (Table 2).

3.2. Biosynthetic Gene Cluster

All Actinomycetotal isolates were selected for the determination of biosynthetic gene sequences of *PKS-I*, *PKS-II*, and *NRPS* by PCR amplification using specific primer sets. The *PKS-II* sequence was detected in 6 isolates (15.8%), while the *PKS-I* and *NRPS* sequences were detected in 20 (52.6%) and 23 (71.9%) strains, respectively (Figures 1-4). The isolate No. 2, which showed a broad-spectrum antimicrobial activity, gave positive amplification products with *PKS-I*, *PKS-II*, and *NRPS* primers. Twelve isolates, including isolates No. 1, 6, 7, and 9 to 20, which exhibited antimicrobial activity against one or two test pathogens, also gave positive amplification products for at least one of the *PKS-I*, *PKS-II*, and *NRPS* genes. Seven (18.4%) isolates neither produced any positive amplification products for the three biosynthetic genes nor showed any antimicrobial activity against the four pathogenic microorganisms that were tested. The 23 antimicrobial-negative isolates still provided positive amplification products for at least one biosynthetic gene (Table 2).

Table 2. Antimicrobial activities and presence of biosynthetic genes in the actinomycetotal isolates

Isolate Code	BGC genes			ISP2				TSB			
	PKS-I	*PKS-II*	*NRPS*	*E. c**	*S. e*	*P. a*	*S. a*	*E. c*	*S. e*	*P. a*	*S. a*
1	+									+	
2	+	+	+						+	+	+
3	+										
4											
5			+								

6	+		+	+						
7			+			+				
8	+									
9	+		+							
10	+									
11	+		+							
12	+		+			+				
13			+			+	+			
14			+			+				
15	+					+				
16	+					+				
17	+	+	+			+				
18	+		+			+		+	+	
19	+		+			+				
20	+	+	+			+				
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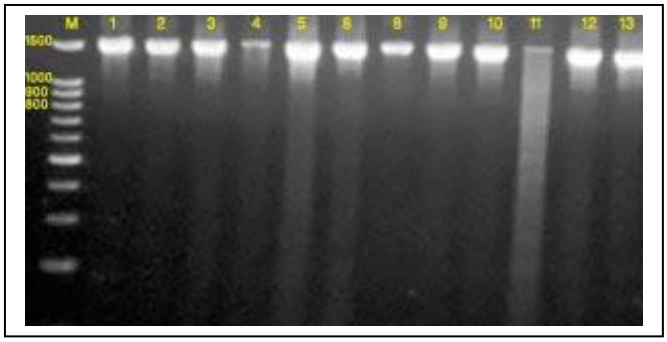

Figure 2. A representative selection of *PKS-I* gene PCR products obtained for actinomycetotal isolates
M= 100 bp DNA size marker
Lanes: 1) Isolate No 1; 2) Isolate No 2; 3) Isolate No 3; 4) Isolate No 6; 5) Isolate No 8; 6) Isolate No 9; 7) Isolate No 10; 8) Isolate No 11; 9) Isolate No 12; 10) Isolate No 15; 11) Isolate No 16; 12) Isolate No 17; 13) Isolate No 18

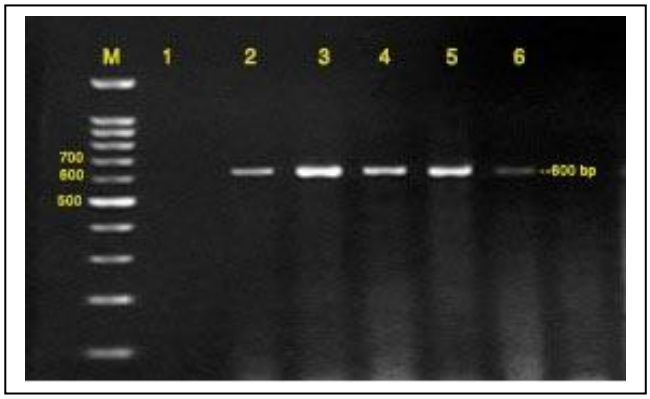

Figure 3. The *PKS-II* gene PCR products obtained for actinomycetotal isolates
M= 100 bp DNA size marker
Lanes: 1) -ve control; 2) Isolate No 2; 3) Isolate No 17; 4) Isolate No 20; 5) Isolate No 23; 6) Isolate No 30

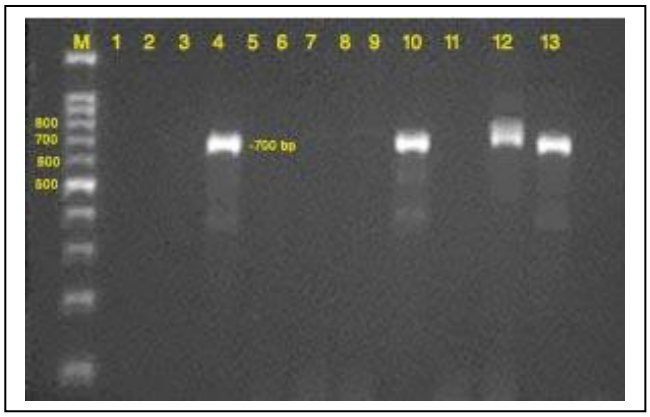

Figure 4. A representative selection of *NRPS* gene PCR products obtained for actinomycetotal isolates
M= 100 bp DNA size marker
Lanes: 1) –ve control; 2) Isolate No 3; 3) Isolate No 4; 4) Isolate No 5; 5) Isolate No 8; 6) Isolate No 10; 7) Isolate No 15; 8) Isolate No 16; 9) Isolate No 21; 10) Isolate No 21; 11) Isolate No 22; 12) Isolate No 24; 13) Isolate No 25

4. Discussion

Endophytic *Actinomycetota* isolated from various medicinal plants represented an enormous reservoir of novel metabolites with potential applications in therapeutics (8-11). This has inspired us to explore the chamomile plant to further understand the endophytic *Actinomycetota* and their biosynthetic potential for producing antibacterial and other useful compounds. In this study, 90 bacterial strains have been isolated from *Anthemis pseudocotula* Boiss. No colonies appeared from the final rinses of the sterilization procedure, demonstrating the effectiveness of surface sterilization and confirming that the subsequent isolates were endophytes. The samples taken in March did not yield any isolates of *Actinomycetota*, but those taken in April and May yielded all of the isolates. This might suggest that older chamomile plants, compared to younger ones, were colonized by *Actinomycetota*. In a study by Cooms and Franco (2003) on wheat roots, the findings suggested that *Actinomycetota* are not present in the roots of young wheat. Previous studies have demonstrated that *Actinomycetota* growth conditions can vary widely in various plants; however, this result differed from that of the current study.

In this study, each isolate was simultaneously inoculated in TSB and ISP2 broth media, and the antibacterial properties of the isolates were examined to show the impact of environmental conditions on the production of secondary metabolites. It is interesting to note that some isolates grown in the TSB medium exhibited antibiotic properties, but not those in the ISP2 medium and that some isolates exhibited this characteristic in the ISP2 environment, but not in the other medium. This phenomenon suggests that there are different chemical constituents in both culture media, and that nutritional factors affect the expression conditions of these genes and the generation of antimicrobial activity. One should seek to create conditions where these genes are expressed in their best state and produce antimicrobial metabolites.

Two antibacterial activity tests (day 7 and day 13) were conducted on the isolates in this study. The number of *Actinomycetota* isolates that exhibited antibacterial properties was much higher in the latter stage. Several factors, including nutrient limitation, growth rate, oxygen or pH conditions, and especially the availability of carbon, phosphate, or nitrogen sources, can strongly affect antibiotic production (21). The production of specialized metabolites in *Actinomycetota* is strictly regulated. Direct regulation

occurs at the level of biosynthetic gene clusters, but frequently in response to how the organism that is producing the biomolecule reacts to its complex and shifting environment. These pathways are frequently activated when *Actinomycetota* are in nutrient-deficient conditions, as demonstrated in experimental studies. The number of *Actinomycetota* with antibacterial properties increased 13 days after the isolates were cultured in a liquid environment, which is consistent with previous findings (21). This is due to the fact that nutrient levels in the cultivation environment decrease over time.

In this study, the strains that were detected as *Actinomycetota* were subjected to molecular evaluation and PCR testing to achieve the next goal in terms of the presence of *PKS-I*, *PKS-II*, and *NRPS* genes. Of the 38 isolated *Actinomycetota*, 23 isolates (60.5%) carried the *PKS-I* gene, 6 (15.8%) possessed *PKS-II*, and 20 (52.6%) harbored the *NRPS* gene. The higher proportions of *PKS-I* and *NRPS* genes, as well as the lower detection rates of *PKS-II*, were in contrast to the results of previous studies (22, 23). In the present study, although the identification of isolates was not performed to the genus level, the higher amplification rates of *PKS-I* were obtained in rare *Actinomycetota* rather than the *Streptomyces*-like organisms. This result was in accordance with the study conducted by another research group (11) in which they claimed that members of the rare *Actinomycetota* showed a higher distribution of the *PKS-I* gene while *PKS-II* was more abundant in *Streptomyces* spp.

There are numerous examples of strains possessing functional genes but not exhibiting antimicrobial activity and vice versa. The percentages of strains with *PKS-I*, *PKS-II*, and *NPRS* genes and the percentage of strains exhibiting antimicrobial activity do not correlate (24, 25). This problem suggests that the expression of *PKS* genes and *NPRS*, as well as the production of microbial secondary metabolites, are highly dependent on environmental conditions.

The endophytic isolates of the present study had detectable antimicrobial activity, and we were able to successfully amplify gene fragments of their genome coding BGC. However, the majority of the inactive isolates had at least one kind of *PKS-I*, *PKS-II*, or *NRPS* genes involved in the backbone biosynthesis of secondary metabolites. These findings showed that the chamomile-associated *Actinomycetota* may be able to produce a variety of secondary metabolites with bioactive properties, in addition to antibacterial ones. We will therefore experiment with various media and culture conditions to promote the expression of secondary metabolite gene clusters in these isolates. Searching the isolates for additional bioactivities, such as anticancer or immunosuppressive activities, are of other plans. To the best of our knowledge, this was the first report on the isolation of endophytic *Actinomycetota* from *Anthemis pseudocutola* Boiss. Therefore, this study may serve as a guide for future research on endophytes, their application, and their potential in the production of bioactive compounds.

Authors' Contribution

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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