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Original Article

Antibacterial activity of metabolites isolated from *Streptomyces* spp. on soil samples of West Azerbaijan province, Iran

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ABSTRACT

Background: The study for new antibiotics is of great importance in investigating programs around the worldwide for pharmaceutical, industrial and agricultural applications. *Streptomyces* like filamentous soil bacteria are used as an essential biological tool for their ability to producing a wide range of new secondary metabolites such as antibiotics. Identification and isolation of new species seemed to be important in the presentation of significantly adequate antibiotics, because antibiotic resistance infectious diseases are the second leading cause of death worldwide, inducing research and development of new antibiotics. Therefore, in this study, we aimed to isolate and characterize novel strains of *Streptomyces* spp. with high antibiotic production ability.

Methods: Soil samples were collected randomly from primitive soils of Urmia, West Azerbaijan province, from Iran in 2019. The isolates of *Streptomyces* spp. were carried out in a specific culture medium. Their primary and secondary antibacterial activity against gram-positive bacteria *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, also gram-negative *Escherichia coli* was checked out. Finally, the antibacterial properties of strains based on *16S rRNA* sequencing were analyzed by MEGA X software.

Results: Totally, 150 colonies were isolated from four soil collected samples. In the primary screening of 10 isolates, insulated antibacterial activity and in the secondary screening, 3 examples were selected. The microorganisms showed antibacterial activity. Sequencing of the *16S rRNA* gene from C-B1-12, D-D3-7, and C-Y2-2 isolates showed similarity to *Streptomyces* indiaensis.

Conclusions: The results of this study indicated that there are new isolates in the soil samples of West Azerbaijan province that are capable of producing new antibacterial agents.

Keywords: *Actinomycetes*, *Streptomyces*, Antibacterial activity, *16S rRNA* gene, Iran

1. Introduction

Recently, resistance to multi-drug in the bacteria has appeared as an important problem that can be a reason for so many diseases in humans, including *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus* sp., etc. [1, 2]. According to the World Health organization in 2001, excessive consumption, global trade, and misuse of

antibiotics led to drug resistance [3]. Hereupon, resistant to available commercially anti-bacterial components increase a need for new molecules with antibacterial properties. Natural sources are most rich in biologically active molecules that are chemically and structurally great diverse and are suggested to find new drugs that can be used against many spectrums of targets [4, 5]. It should be noted that, the secondary metabolites of microbes are the useful source for finding components of biological activities [6].

Actinomycetes are known as antibiotics, particularly Streptomyces spp. produce antibacterial compounds, secondary metabolites and enzymes, which are important in medicine. Accordingly, about 75% of commercial antibiotics products by Streptomyces species [7, 8]. They are prominent as gram-positive, high CG%, aerobic, and mycelial bacteria [9, 10].

Soil as their main habitat is rich in nutritionally, thereupon, they can carry out a wide range of biological processes and produce extremely varied bioactive secondary metabolites [11]. The *Streptomyces* is a principal genus in the industrial applications, human medical health, ecology, biotechnology, and studying evolution of bacteria [12]. Hence, isolation, identification, and survey of antibacterial activity of promising strains of *Streptomyces* spp. producing antibacterial component have been the main focus of research for many years [13].

In the present study, isolation, identification, and investigation of *Streptomyces* spp. producing secondary metabolites of antibacterial activity isolates from soil sample of west Azerbaijan province and their antagonistic properties against *S. aureus*, *B. cereus*, *L. monocytogenes*, and *E. coli* have been explained.

2. Materials and Methods

2.1. Materials

The *Bacillus cereus* (ATCC 1431), *Staphylococcus aureus* (ATCC 29231), *Listeria monocytogenes* (ATCC 33090) and gram-negative *Escherichia coli* (ATCC 1399) bacterial strains were provided from Faculty of Veterinary Medicine, Islamic Azad University, and Urmia, Iran. Starch Casein Agar (SCA), International *Streptomyces* Project-2 Medium (ISP-2), Muller Hinton Agar (MHA) was obtained from Q-Lab Company (Q-Lab Company, USA). Chemical Compounds such as Dichloromethane (Dic), Ethyl acetate (Et), Hexane (H), Chloroform (Co), Methanol (M), Diethyl ether (Die) and Dimethyl sulfoxide (DMSO) were purchased from Merck (Merck, Germany). All bacteria culture plates, tips and falcon were obtained from SPL Life Sciences (Anyang, Korea). All other chemicals not mentioned above for DNA extraction and gel electrophoresis were provided by Takapouzist (Takapouzist, Iran) and Sinaclon (Sinaclon, Iran), Starch Casein Agar (SCA), International Streptomyces Project-2 Medium (ISP-2), Muller Hinton Agar (MHA), Dichloromethane (Dic), Ethyl acetate (Et), Hexane (H), Chloroform (Co), Methanol (M), Diethyl ether (Die), water (DDW), Muller Hinton Broth (MHB), Dimethyl sulfoxide (DMSO).

2.2. Sample collection

Soil samples were collected in the 2019 from Targever, Noushan, Sheyban and Razhan village in Urmia, West Azerbaijan province, Iran at 20cm in depth and 25cm in diameter, then they were kept at 4°C for the next analyses [14].

2.3. Isolation of *Streptomyces* spp.

To prepare soil suspension, mixed 5 g of soil sample with 45 mL of sterile

distilled water and shacked for 30 min, then pH value was measured. Soil suspensions were serially diluted and, the third concentration of 100 µL was used to inoculate SCA (Q-Lab Company, Ohio, and USA) and was incubated at 28 ˚C for seven days. Two hundred sixteen colonies were selected, and for specific isolation of *Streptomyces* spp., these bacteria were cultured in an ISP-2 (Q-Lab Company, Ohio, and USA). Morphological characteristics of colonies including number and pigmentation colony recorded for primary classification of bacteria and were stored at -20 ˚C. Each colony was tagged based on sampling location, plate number and colony number [15, 16].

2.4. Antimicrobial activity

In this experiment, testing organisms included: gram-positive *Bacillus cereus* (ATCC 1431), *Staphylococcus aureus* (ATCC 29231), *Listeria monocytogenes* (ATCC 33090) and gram-negative *Escherichia coli* (ATCC 1399). The overlay and disk diffusion methods [17] were used for antibacterial properties of *Streptomyces* spp. isolates in the primary and secondary screening of antimicrobial activity, respectively.

The primary screening of *Streptomyces* spp. antimicrobial activity against indicator bacteria was determined by using the overlay method. In this method, *Streptomyces* spp. isolated from soil were cultured in SCA medium and then microorganisms were cultured in MHA (Q-Lab Company, Ohio, and USA) media for preparation suspensions. After preparing the suspension, they are mixed (with a ratio of 2/3) with MHA culture medium that contains the indicator strain (e.g., *S. aureus*). The inhibitory effect was investigated after ten days of incubation at 37 °C. The area of inhibition was measured using a millimeter scale [18, 19].

The antimicrobial activity of extracted secondary metabolites was by different solvents such as Dic, Et, H, Co, M, Die (Merck, Darmstadt, Germany), and water (DDW) evaluated by disc diffusion method. To do this step, antibacterial metabolites of the primary screening positive isolates were selected. For extracts preparation, the microorganism's selection was inoculated into 100 mL in MHB (Q-Lab Company, Ohio, and USA) and incubated for 36h at 29 °C with 125 rpm. The culture medium was centrifuged (Sigma, Dorset, United Kingdom) at 4000 rpm for 20 min, then the suspension was collected and mixed by various solvents. The solutions were shaken for about one hour at 29 °C at 175 rpm; next, the liquid phase was separated from the solvent phase and concentrated to 7 mL using a rotary evaporator (Heidolph, Schwabach, Germany). Blank disks of 6mm diameter were immersed in Secondary metabolites. All indicator bacteria cultured in MHA media and the blank discs, including secondary metabolites of each isolate were put on them. After 15 min all plates were held back incubated in a 37 °C for 24 h. The area of inhibition around the blank disk was measured and recorded. DMSO was used as solvent control, and streptomycin was used as a reference control for indicator bacteria [20, 21].

2.5. DNA Extraction of *Streptomyces* spp.

To genomic DNA extraction, streptomycetes isolated in ISP2 medium were cultured for three days at 30 °C. After the incubation, the culture was centrifuged at 10000 rpm for 5 minutes; the supernatant was discarded and washed twice with double-distilled water. The genomic DNA of *Streptomyces* species was isolated using a previously published protocol [22]. To amplify of *16S rRNA* gene by using PCR with Taq

DNA polymerase and universal primers used were as follows: 5/AGAGTTTGATCCTGGCTCA3/ (forward) and 5/AAGGAGGTGATCCAGCCGC3/ (reverse) [23]. PCR amplification was carried out using Thermal Cycler (Analytik Jena AG, Germany), the final volume of 50 µL including 25 µL Master Mix (Cinnagene, Iran), 60 ng/mL chromosomal DNA, 10pmol of each primer. The PCR conditions contained denaturation at 95 ˚C for 10 minutes followed by 40 cycles of denaturation at 95 ˚C for 30 seconds, primer annealing at 55 ˚C for 30 seconds and primer extension at 72 ˚C for 2 minutes and a final extension at 72 ˚C for 10 minutes. The PCR products were visualized by 1.0% (w/v) agarose gel containing red safe and DNA ladder marker with 1 kb was used compared with the ultraviolet fluorescence gel documentation system (UVITEC, England, United Kingdom).

2.6. Sequencing and phylogenetic analysis

The PCR products were sequenced by the Takapouzist Co (Tehran, Iran). Furthermore, the sequences analysis of the *16S rRNA* of *Streptomyces* species were contrasted with the reference different species of bacteria contained in the GenBank database, using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov). Molecular Phylogenetic analysis was done by Maximum Likelihood method based on the Tamura-Nei model [24]. All the analyses were conducted on a bootstrap dataset containing 1000 replicates. Evolutionary analyses were performed in MEGA X software [25, 26].

3. Results

3.1. Sample collection and isolation of *Streptomyces* spp.

Totally 16 soil samples were collected from 4 different locations within four districts along the Urmia County, West Azerbaijan Province, Iran (Targever, Sheyban, Noushan, and Razhan villages) (Figure 1A). Four samples were collected from each region. The pH volume soil was recorded in the second dilution (Figure 1B). In total, 216 colonies were selected for antibacterial properties evaluation on the SCA medium (Figure 2) (Table 1).

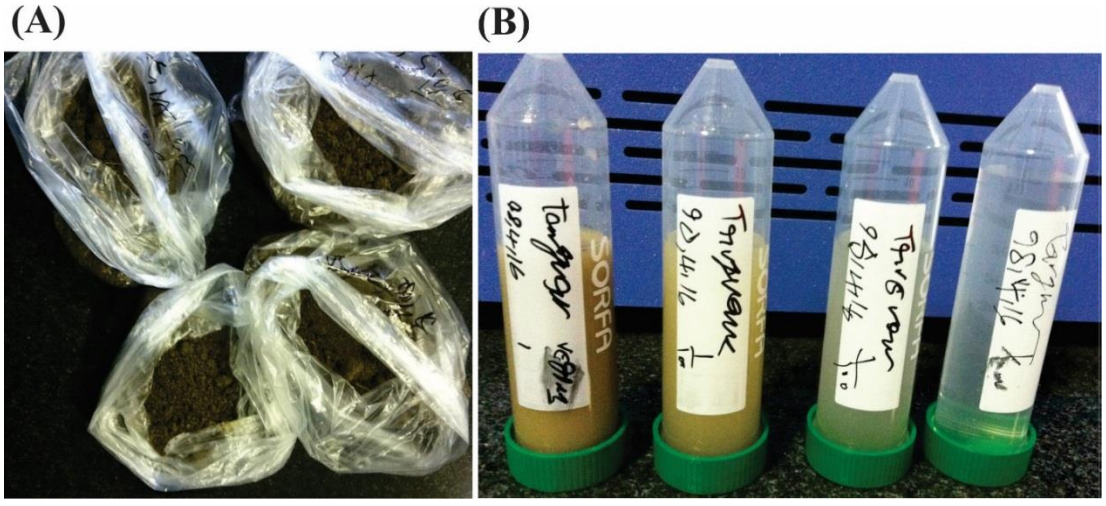

Figure 1. (A) Soil samples collected from Targavar area; (B) Serial dilution using soil sample

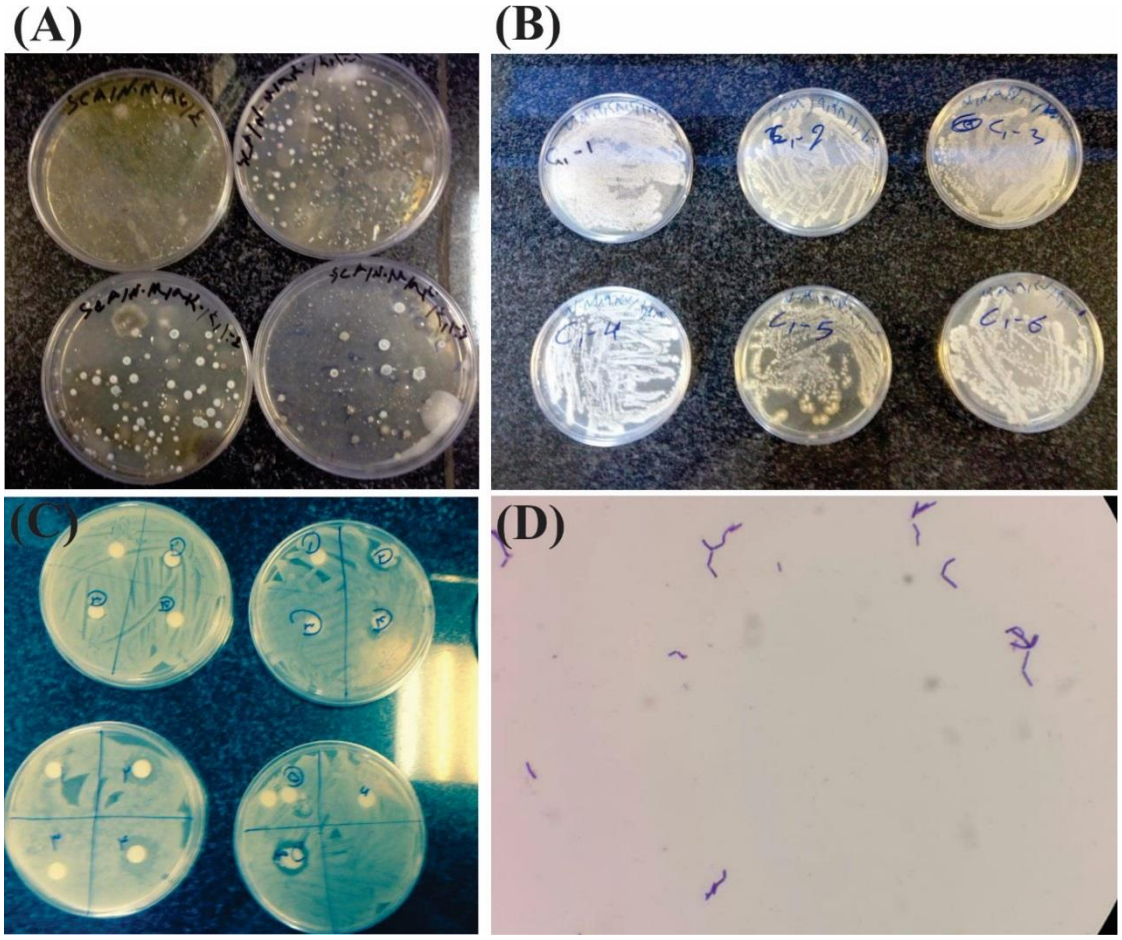

Figure 2. Isolation plates from soil samples of Urmia County, West Azerbaijan Province, Iran. (A) Actinomycetes strain identified from soil sample and the growth was observed after 7 days, (B) 216 colonies were selected and for specific isolation of *Streptomyces* spp., these bacteria were cultured in an ISP-2 medium, (C) Disk diffusion methods were used for antibacterial properties of *Streptomyces* spp. isolates in the primary and secondary screening of antimicrobial activity and (D) Morphological features of isolates under light microscope after Gram's reaction were used (100x)

Table 1. Primary characteristic of soil samples from Urmia County, West Azerbaijan Province, Iran

Sampling area	Number of samples	Soil code	pH volume	Number of colonies
Targever	4	A	7.7	54
Sheyban	4	B	7.64	34
Noushan	4	C	7.71	22
Razhan	4	D	749	106

3.2. Preliminary screening bioassays

The 216 colonies isolated in the media of ISP2 were cultivated, of which six colonies showed potent antimicrobial properties against the indicator bacteria, and four colonies were sensitive (Table 2). The pigment properties produced by these ten bacteria are shown in Table 3.

3.3. Secondary screening bioassays

Approximately 30% of the selected isolates appeared in the primary screening showed antibacterial activity. Secondary metabolites extracted from which organic solvents were evaluated using the disc diffusion method. It should be noted that the *L. monocytogenes* bacteria did not respond to any solvents and the extracted metabolites of dichloromethane showed the most antibacterial activity (Table 4).

Table 2. Inhibitory effect of isolated bacteria against some selected index bacteria

Sample Code	*Escherichia coli* (ATCC 1399)	*Bacillus cereus* (ATCC 1431)	*Staphylococcus aureus* (ATCC 29231)	*Listeria monocytogenes* (ATCC 33090)
A-U1-1	+	+	+	+
C-B1-12*	-	-	-	-
B-M2-2	+	+	-	+
D-I2-1	+	+	+	+
C-Y2-2*	-	-	-	-
B-M2-1*	-	-	-	-
D-D3-7	+	-	+	+
A-Z2-1	+	+	-	+
D-D3-6*	-	-	-	-
A-M1-1	-	+	+	+

*Colonies are sensitive

Table 3. Pigment properties of selected *Streptomyces* spp. isolates with high antibacterial activity collected on soil samples from Urmia County, West Azerbaijan Province, Iran

Sample Code	Colonies pigmentation	
	Surface	Back
A-U1-1	White and chalky	cream
C-B1-12	Milky	Milky
B-M2-2	Brown	Dark
D-I2-1	White	cream
C-Y2-2	White and chalky	White
B-M2-1	Phosphoric	Dark
D-D3-7	White	Milky
A-Z2-1	Brown	Dark
D-D3-6	White and chalky	White
A-M1-1	Brown	Dark

Table 4. Secondary antimicrobial activity of ten potential *Streptomyces* spp. against *Listeria monocytogenes*, *Bacillus cereus, Staphylococcus aureus* and *Escherichia coli*

Isolate	Indicator bacteria	Dic*	Et*	H*	Co*	M*	Die*	DDW*
A-U1-1	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
C-B1-12	*E. coli*	+	-	-	-	-	-	-
	S. aureus	+	+	-	-	+	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
B-M2-2	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
D-I2-1	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
C-Y2-2	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	+	+	+	+	+	-	-
B-M2-1	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
D-D3-7	*E. coli*	-	-	-	-	-	-	-
	S. aureus	+	-	+	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
A-Z2-1	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
D-D3-6	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-
A-M1-1	*E. coli*	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-
	L. monocytogenes	-	-	-	-	-	-	-
	B. cereus	-	-	-	-	-	-	-

*Dic (Dichloromethane), Et (Ethyl acetate), H (Hexane), Co (Chloroform), M (Methanol), Die (Diethyl ether) and DDW (Water)

3.4. PCR and Phylogeny

For molecular identification of three isolates with the highest antibacterial activity in secondary screening, PCR was performed by AS-F and AS-R primers. In all three isolates, only a 1,500 bp band was observed (Figure 3). The alignment of the nucleotide sequencing (1500 pb) of tree isolates (C-B1-12, D-D3-7, and C-

Y2-2) in the gene bank using NCBI and the relationship between *Streptomyces* spp. strains were evaluated by MEGA X software to compare the DNA sequencing generated by each primer represented D-D3-7 and C-Y2-2 high similarity with *Streptomyces indiaensis* (Kudo Seino, 1987) and *Streptomyces* sp strain BD99, respectively. However, based on this finding, C-B1-12 can fall into clusters whose strains have not yet been identified (Figure 4). The isolates C-Y2-2 and D-D3-7 produced similar patterns, so they were categorized as the same strain. The isolate C-Y2-2 was the promising component with high antibiotic production capacity, because it showed antibacterial activity in five different conditions. Although C-Y2-2 and D-D3-7 were in the same cluster, D-D3-7 showed the least antibacterial activity among the isolated strains. The isolates C-B1-12 with showed four condition antibacterial could be a good option for future studies.

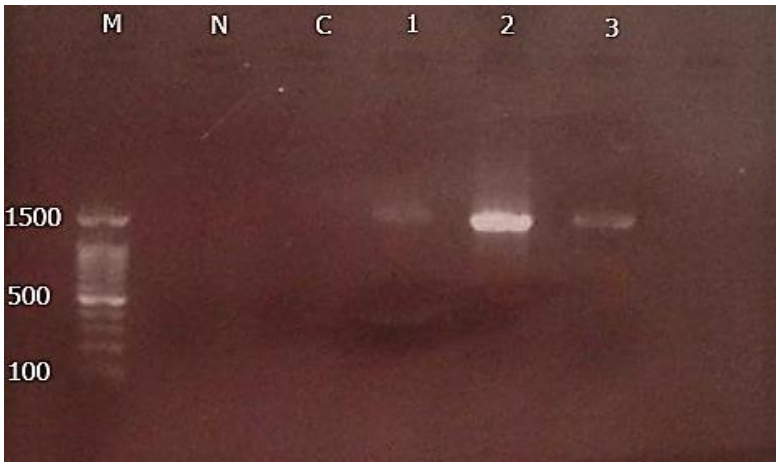

Figure 3. Amplification of the *16S rRNA* gene, using PCR for three highly active bacteria. Amplified PCR products were electrophoresed on an agarose gel (1%). The symbols in PCR lanes represent: M: Marker (100 bp); N: Null; C: Negative control; 1: C-B1-12; 2: C-Y2-2; 3: D-D3-7

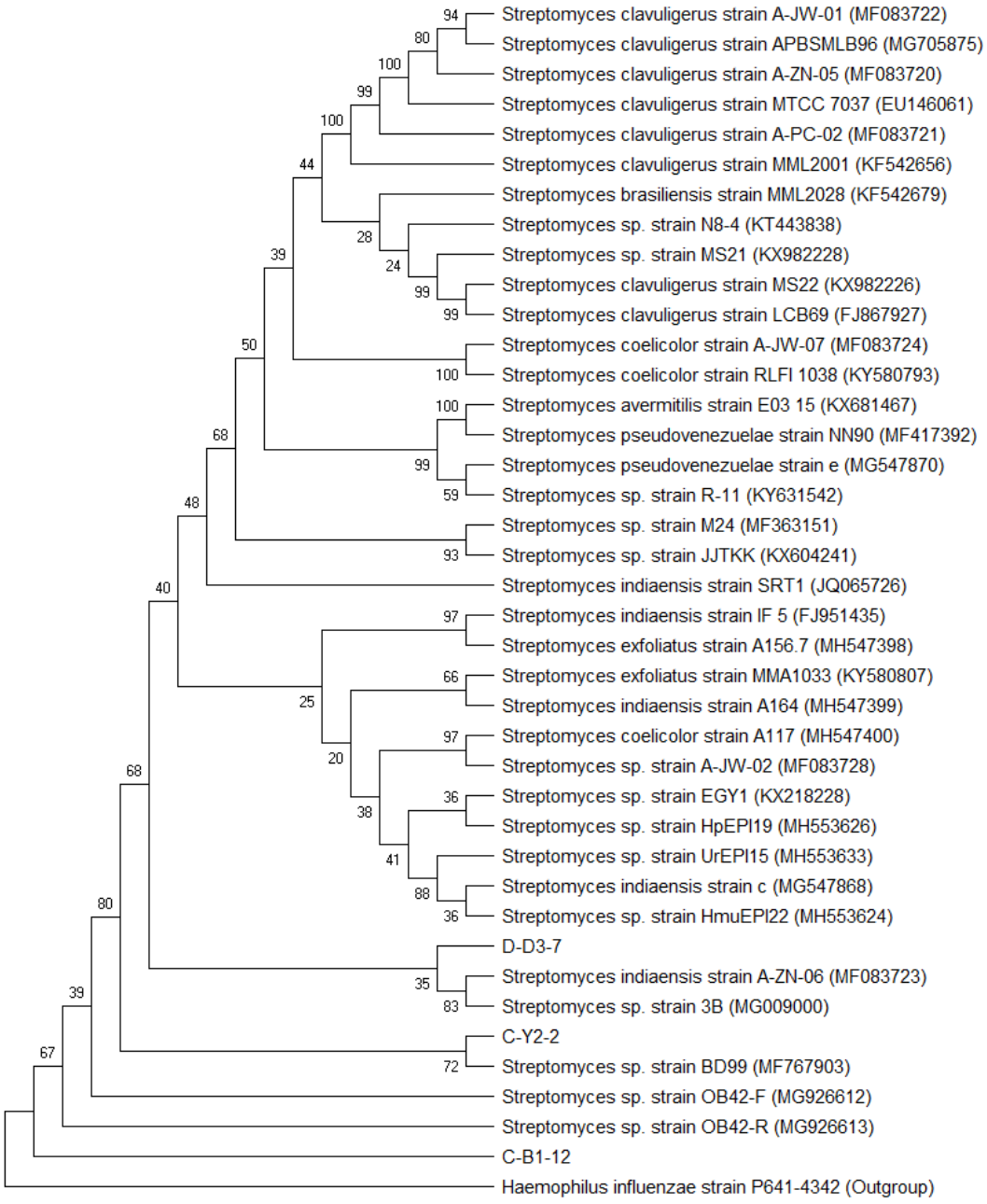

Figure 4. *16S rRNA* tree showing the phylogenetic relationship by Maximum Likelihood method based on the Tamura-Nei model between three isolates with other known *Streptomyces* species

4. Discussion

Despite great success in finding new drug molecules with antibacterial properties and new techniques developed in their manufacturing process, mortality rates due to infectious diseases is still high worldwide [27]. One of the main reasons for these issues is multidrug resistance among the pathogenic microbes, which creates the field to search for new and potential bioactive molecules [28]. Interestingly, the bacteria themselves act as novel drug source. Cyclic lipopeptide purified from *Paenibacillus ehimensis* with antibacterial properties act against of Pseudomonas

aeruginosa [29]. 1-methyl ester-nigericin isolated from *Streptomyces hygroscopicus* has an antibacterial activity [30]. Therefore, it is increasingly essential to study the resistance of pathogens to antibiotics. Accordingly, investigating the habitat of living organisms with antibacterial products is of great importance. Soil is a rich environmental resource where many organisms live together, and some produce useful natural products with antibacterial properties. In this study, we tried to identify the recognized strains of *Streptomyces* spp. with excellent efficiency of antibiotic production from several samples of soil in west Azerbaijan of Iran using molecular techniques. Distinguished factors were used, such as soil pH, morphological characters like colonies, and pigment colors. Also, molecular method was used *16S rRNA* gene. Although the sequence of the *16S rRNA* gene has not changed much in the evolution process, it is 5' variable region including α, β, γ, δ, and ε are more simple and yet efficient for identification of new *Streptomyces* spp. strains [31]. Therefore, they have a special place in classification studies and we used genetic diversity studies of different *Streptomyces* species strains.

Over the years, for the screening of bioactive compounds to isolate of novel antibiotics, thousands of *Actinomycetes*, especially *Streptomyces* species strains, are screened each year by pharmaceutical laboratories as a source of new antimicrobial compounds. In the present study, the isolate exhibited wide antimicrobial activities against Gram-positive and Gram-negative bacteria in the primary and secondary screening process; however, there was no correlation between the activity of intact bacteria and secondary metabolites. Isolates that were active in their preliminary screening, but were inactivated in secondary screening were tested with experimental microorganisms (Table 4). One reason to justify this phenomenon may be the lack of proximity of pathogens to these isolates and the loss of competitive space. Contrary to this, results were obtained for isolates C-Y2-2 and C-B1-12, which had no significant primary screening activity but were the most active isolates in the secondary screening. Similar results were observed by Pandey et al. (2004) which studied Nepalese Actinomycetes. A study conducted by Oh et al. (2005) distinguished a new strain of *Streptomyces* species with high antibiotic production ability and increased homology to *S. echinatus* by the evaluation of cultural, phylogenetic assessment determinants used *16S rRNA* sequence analysis [12, 32, 33]. In a similar study, Higginbotham et al. (2010) identified two strains with very homology by *S. lavendulae* and *S. globosus* using RNA sequencing [34].

5. Conclusions

From this study, we identified some isolates with high antibacterial activity. However, further research is needed to determine the active metabolites of these isolates. It should be noted that by designing appropriate strategies, ideal results can be obtained. For example, the importance of glucose in the nutritional media for the synthesis of a wide range of antibiotics by different *Streptomyces* species has been reported by many researchers [35, 36]. Therefore, using media culture containing different concentrations of glucose can show the strains present with higher antibacterial activity. Also, optimized culture condition in the different levels including pH, temperature, and time, even can be applied. Therefore, we need further studies in other situations, and isolation of antimicrobial compounds from the culture medium introducing novel and highly effective conceivable antibiotics.

Conflict of interest

The authors declare that they have no competing interests.

Consent for publications

Not applicable.

Availability of data and material

Not applicable.

Authors' contributions

Study concept, design, data were analyzed, interpreted, drafting of the manuscript and critical revisions of the manuscript for important intellectual content were done by MRA and NM. All authors read and approved the final manuscript.

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Ethics approval and consent to participate:

Not applicable.

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