



Research Article

Identification and Antimicrobial Activity of Actinobacteria Isolated from Rhizosphere of the Local Endemic Plants

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Abstract: The search for novel antimicrobial compounds from the environment has been gaining momentum with the increase in resistance of pathogens. In the present study, the antimicrobial potential of 11 local isolates of Actinobacteria which were isolated from rhizosphere soils of the three local endemic plants in Turkey was evaluated against different pathogenic bacteria and *Candida albicans*. Antimicrobial activity was investigated by the disk diffusion agar method using the organic extracts obtained from the isolates grown in the modified Bennett's medium and Tryptic Soy Broth (TSB). Eight isolates were revealed to show remarkable antimicrobial activity against to pathogens. Study of the production of bioactive metabolites in two different culture media indicated that the higher antimicrobial activity was observed in modified Bennett's medium when compared to TSB. Also, partial 16S rDNA sequence analyses revealed that 10 of these local isolates belong to member of *Streptomyces* and one of them to member *Promicromonospora* genera.

Lokal Endemik Bitki Rizosferinden İzole Edilen Aktinobakterilerin Tanımlanması ve Antimikrobiyal Aktivitesi

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Anahtar kelimeler

16S rRNA,
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Moleküler tanımlama,
İkincil metabolitler.

Öz: Patojenlerin direncinin artmasıyla birlikte yeni antimikrobiyal bileşik arayışı hız kazanmaktadır. Bu çalışmada, Türkiye'deki üç yerel endemik bitkinin rizosfer topraklarından izole edilen 11 lokal Aktinobakteri izolatının farklı patojen bakterilere ve *Candida albicans*'a karşı antimikrobiyal potansiyeli değerlendirilmiştir. *In vitro* antimikrobiyal aktivite, modifiye Bennett's ve Tryptic Soy Broth (TSB) besiyerinde üretilen izolatların organik özütleri kullanılarak disk difüzyon agar yöntemiyle araştırılmıştır. Sekiz izolatın patojenlere karşı kayda değer antimikrobiyal aktiviteye sahip olduğu belirlenmiştir. Biyoaktif metabolitlerin üretimi üzerine iki farklı kültür ortamının incelenmesi sonucu, modifiye Bennett ortamında daha yüksek antimikrobiyal aktivitenin elde edildiği bulunmuştur. Ayrıca kısmi 16S rDNA dizi analizleri ile bu lokal suşların 10'unun *Streptomyces* ve birinin de *Promicromonospora* cinsine ait olduğu tespit edilmiştir.

1. Introduction

The phylum Actinobacteria are considered to be one of the top producers of secondary metabolites, such as antibiotics, immunosuppressors, antifungals and antitumoral, etc. (Ay, 2020; Kämpfer and Labeda, 2006). The *Streptomyces*, which is the largest genus of Actinobacteria, consists of almost 800 species with published names (www.bacterio.net/streptomyces.html). In recent years, as a result of the new generation sequencing analyses into the Actinobacterial systematics, many reclassifications have been made within the genus *Streptomyces* (Nouioui et al., 2018). However, 16S rRNA sequence data still are valuable in the systematics of this genus (Fguira et al., 2005).

One of the important features of the *Streptomyces* genome is the presence of gene clusters responsible for the synthesis of crucial secondary metabolites (Nett et al., 2009). *Streptomyces* genus has been an important reservoir of medically important metabolites, particularly antibiotics. Since the first antibiotic discovery, many antibiotics have been isolated and described from various *Streptomyces* species (Berdy, 2005). The great importance of *Streptomyces* species is due to the urgent need to discover new antimicrobial compounds because of the rapid increase of multi-drug-resistant microbial pathogens (Bush et al., 2011). For this reason, *Streptomyces* has become the main focus of isolation and investigation from different habitats including plant root environment (Yilmaz et al., 2008). Moreover, the large amount of information gathered about *Streptomyces* species makes them more amenable to the application of -omic techniques to unlock their potential to produce potent bioactive metabolites (Nett et al., 2009). Therefore, systematic investigation of *Streptomyces* species becomes more important as they still have the potential to produce many undiscovered secondary metabolites.

This study aimed to determine the variety of Actinobacteria isolated from the rhizosphere soils of three endemic plants, *Aethionema dumanii*, *Salvia aytachii*, and *Achillea ketenoghui* from Beypazarı, Turkey, by 16S rRNA reading. Also, in vitro investigation of antimicrobial activities of isolates grown in two different test media by the disk diffusion agar method is another achievement of this study.

2. Material and Methods

2.1. Actinobacterial isolates and test organisms

Bacteria were previously isolated from the alkaline root surrounding soil of *A. dumanii*, *S. aytachii*, and *A. ketenoghui* endemic plants near Ayaş-Beypazarı (Ankara), Turkey (Yilmaz et al., 2008).

In the study of evaluation of antimicrobial properties of the ethyl acetate extracts obtained from the isolates grown in the modified Bennett's and Tryptic Soy Broth (TSB) media, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231) were used as the indicator microorganisms for antimicrobial activity assay. All strain cultures were grown either in Nutrient Broth (NB) broth (Difco, Detroit, MI, USA) or on Nutrient Broth agar plates at 25 °C. Sabouraud Dextrose Agar (SDA, Merck) or broth was used for *C. albicans* production.

2.2. Genomic DNA isolation and PCR

TSB medium with 0.5% maltose was used to grow the isolates using an orbital shaker (230 rpm) for 48 hours. 2 ml of it was transferred to a liquid medium containing TSB-YEME and left to incubate at 28 °C for 24 hours in the orbital shaker (230 rpm). 1.5 ml culture was centrifuged and washed twice using 10.3% (w/v) sucrose solution. Pellet was dissolved in 500 µl TSE buffer (25 mmol l⁻¹ Tris– HCl pH 8.0, 300 mmol l⁻¹ sucrose, 25 mmol l⁻¹ EDTA) supplemented with 2 mg ml⁻¹ lysozyme and 50 µg ml⁻¹ RNase and incubated at 37 °C for 30 min. 300 µl 2% (w/v) SDS was added and vortexed for 20 s. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) solution was used for extraction, and it was followed by another extraction step with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). In the last step, the DNA in the supernatant was precipitated by standard precipitation method using sodium acetate (3M).

2.3. Genotypic characterization of the isolates

The isolates were identified by partial 16S rRNA gene sequencing. The genes were amplified in a polymerase chain reaction (PCR) using the chromosomal DNA as a template and universal bacterial primers, 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GTTACCTTGTTACGACTT-3'). A 50 µl reaction mixture contained 2.5 U Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.3 mM dNTPs, 25 mM MgCl₂, 20 pmol of each primer, 5 µl of 10x reaction buffer (Thermo Fisher Scientific), and 100 ng of template DNA. The step-up PCR procedure included denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were run on a 1 % agarose gel. DNA of the expected size was purified from the gel with the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

16S rRNA gene regions were performed by İontek Biotechnology Company (Turkey) using the chain termination method with Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham), using a Perkin Elmer-ABI Prism 377 automated sequencing platform. The sequences obtained were analyzed using the database on the website "https://www.ezbiocloud.net/", and then the sequences were logged in to the GenBank site and accessed accession numbers. The phylogenetic tree was constructed by the GGDC web server at <http://ggdc.dsmz.de/> using the phylogenomic data line DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) adapted to single genes (Meier-Kolthoff et al., 2013). Multiple sequence alignment was done with "MUSCLE" (Edgar, 2004), and the phylogenetic tree was created using the Maximum Likelihood method (Stamatakis, 2014).

2.4. Extraction of crude metabolites

Each of 11 isolates was inoculated aseptically into 50 ml Bennett's medium in baffled flasks (1 g yeast extract (Fluka), 1 g beef extract (Acumedia), 2 g bacto casein (Difco), and 10 g glucose per l in tap water, pH 7.8) or TSB (Oxoid) media. They were incubated for 3–5 days at 28°C in a rotary shaker at 250 rpm. Then, 500 µl of liquid cultures were inoculated in 50 ml media again and then left for incubation for three days.

After three days of incubation, a press was used to separate the mycelium and supernatant of the culture. The supernatant was extracted with ethyl acetate (EtOAc) (2 x 100 ml). Both extracts were combined and dried with Na₂SO₄. Crude extracts were obtained by evaporation to dryness under reduced pressure and weighed. Then they were dissolved in ethyl acetate (EtOAc) and used for antimicrobial screening.

2.5. In vitro antibacterial activity

The antibacterial activity of the extracts was evaluated using the disk diffusion method (NCCLS, 2002). Bacterial and *C. albicans* suspensions were prepared with the spectrophotometric method. The final bacterial and *C. albicans* concentration applied on the agar surface was approximated to 10⁷ CFU and 10⁴, respectively. The disk diffusion method was performed using Nutrient Agar (NA, Oxoid) for bacteria and Sabouraud Dextrose Agar (SDA, Oxoid) for *C. albicans*. Sterile test discs (Bioanalyse Co., Ltd., Ankara, Turkey) were placed on the agar surface, which had been previously inoculated with 0.1 mL bacterial suspension at an equal distance from each other. 20 µL of each extract was directly applied onto the disks, and the plates were left for incubation at 37°C. Inhibition zones were measured after 24 hours for bacteria and 48 hours for *C. albicans*. Antibiotic discs (all from Oxoid) containing imipenem (10 µg disc⁻¹), ofloxacin (5 µg disc⁻¹), netilmicin (30 µg disc⁻¹), amoxicillin (30 µg disc⁻¹), erythromycin (15 µg disc⁻¹), and Amphotericin B (20 µg disc⁻¹) were used to test the antibiotic susceptibility. EtOAc was used as a negative control for each experiment in the disk diffusion method. The presence of a clear zone around the disks was considered as inhibition.

3. Result and Discussion

The rapid spread of antibiotic resistance among pathogens has created an urgent need for drugs. Bioactivity-guided screening of the novel secondary metabolites from microorganisms may provide the potential to overcome the antimicrobial drug-resistance, which is a great public health problem (WHO,

2014; Schein, 2020). In the present study, it was aimed to screen the rhizospheric Actinobacteria to determine the effective anti-microbial strains against pathogen organisms.

Partial (300-600 bp) 16S rRNA gene sequencing of isolates yielded 10 *Streptomyces* and 1 *Promicromonospora* genera according to homology search using the EZBioCloud database (Table 1). The full-length 16S rRNA gene contains nine variable regions (V1-V9) distributed throughout the highly conserved sequence. Our partial sequences (300-600 bp) cover the V1-V3 variable regions (Johnson et al., 2019). Since modern analysis sequencing methods provide an appropriate read of full-length of the 16S rRNA gene, they have the potential to give a better taxonomic analysis of bacterial communities at genus, species, and even strain levels. However, it was also shown that although the full V1-V9 region consistently produced the best results, some sub-regions sequencing produced very good results for classifying sequences for some genera. For example, the V1-V3 region gave satisfactory results for *Escherichia/Shigella* (Johnson et al., 2019). Taxonomic analysis based on the initial 120-bp sequences has been demonstrated in some previous studies to be useful for the identification of *Streptomyces* species (Kataoka et al., 1997). Also, Yilmaz et al., (2008) constructed phylogenetic trees by the neighbor-joining method in their study using both full length and partial 16S rRNA sequences of *Streptomyces* isolates and representative strains. They found that the general topologies of trees from both sequences were similar. On the other hand, genetic distance was more pronounced in the partial-sequence tree.

Table 1. Identification of isolates and GenBank accession numbers according to the results of sequence analysis using the EzBioCloud database

Code of the Isolates	Top-hit Taxon	Top-hit Strain	Similarity (%)	Completeness (%)	GenBank Accession Numbers
AR9	<i>Promicromonospora xylanilytica</i>	YIM 61515	99.50	27.7	MT741827
CS41	<i>Streptomyces chryseus</i>	NRRL B-12347	99.03	21.4	EF494233
CA12	<i>Streptomyces spiroverticillatus</i>	NBRC 12821	98.33	20.7	MT738695
BS32	<i>Streptomyces seymenliensis</i>	B1041	98.63	35.2	MT733233
BS29	<i>Streptomyces hypolithicus</i>	HSM10	99.71	23.3	MT733215
BA14	<i>Streptomyces peucetius</i>	JCM 9920	98.78	22.6	MT733216
BA12	<i>Streptomyces xiangluensis</i>	NEAU-LA29	99.03	21.3	MT733214
AS36	<i>Streptomyces narbonensis</i>	NBRC 12801	99.67	41.7	MT733200
AS28	<i>Streptomyces chryseus</i>	NRRL B-12347	99.73	25.7	MT733199
AR6	<i>Streptomyces flavofungini</i>	NBRC 13371	99.49	41.0	MT733185
AR4	<i>Streptomyces shaanxiensis</i>	CCNWHQ 0031	99.36	21.6	MT733183

The 16S rRNA gene sequencing results of all our isolates revealed 98-99.73% similarity rates (Table 1). Stackebrandt and Goebel, (1994) proposed the 97% 16S rRNA sequence similarity threshold to confirm that the two strains do not belong to the same species. Then, 98.7% sequence similarity ratio was suggested as an originality value, which is higher than the 97% threshold value (Stackebrandt and Ebers, 2006). After a few years, it was proposed that a 98.65% similarity ratio can be used as the threshold of distinguishing two species (Kim et al., 2014). Except for isolate BS32, the 16S rRNA gene sequencing results of all our isolates yielded a higher than 98.65% similarity rate. The similarity value of the isolate BS32 (98.63%) which was related to *Streptomyces seymenliensis* is very close to this threshold (Figure 1). Therefore, it can be stated that our partial 16S rRNA sequences did not yield a new species. It should be noted, however, that although the 16S rRNA gene sequence is a strong indicator, it may not be sufficient alone to identify a new species. A polyphasic approach that encompasses phenotypic and chemical characterizations as well as whole genome-based analysis should be adopted to precisely determine the taxonomic position of a strain (Tindall et al., 2010).

Antimicrobial activities of 11 identified isolates that were grown in modified Bennett's medium and TSB are shown in Table 2. Table 3 shows the results of the antibacterial test of some of the standard antibiotics against tested microorganisms. It was shown that the crude EtOAc extracts of 8 of the isolates had antimicrobial activity against different test organisms. Among them, extracts of CS41 isolate grown in modified Bennett's medium were found to have a strong inhibitory effect on Gram-positive organisms. BS29 grown in the same medium was the only isolate showing both antibacterial and antifungal activity. No antimicrobial effect of the majority of the extracts from isolates grown in TSB medium was observed against test microorganisms. On the other hand, EtOAc extracts of isolates grown in modified Bennett's medium showed remarkable antimicrobial activity (Table 2). According to the results, it was observed that the antibacterial effects of the extracts were more prominent on Gram-positive bacteria compared to Gram-negative bacteria. These findings are parallel to the observations made by the other researchers in their studies in the literature (Basilio et al., 2003; Saadoun and Gharaibeh, 2003; Sahin, 2005). This might be explained by the difference in cell wall compositions between the two groups. The more peptidoglycan structure in the cell wall of Gram-positive bacteria makes these bacteria more sensitive to antibacterial agents, especially antibiotics (Yilmaz et al., 2008).

Also, various factors such as pH, temperature, minerals, and culture media components affect secondary metabolite production. Some of the 11 isolates grown in Bennett+ Glucose medium had antimicrobial activity. On the other hand, they did not show any activity when they were grown in TSB medium. The difference in antimicrobial activity due to the factors mentioned above shows how important the medium is in this regard. The three isolates BA12, BA14, and BS29 were also found to have antimicrobial activity against *E. coli* and *C. albicans* when grown in modified Bennett's medium. Many studies have shown that the role of nutrients and the environment have very important effects on the amount and variety of secondary metabolite synthesis (Bills et al., 2008; VanderMolen et al., 2013). Different sources of carbon and nitrogen in the culture medium can increase or decrease the synthesis of a bioactive secondary metabolite through different ways, such as enzyme induction or inhibition. There are studies indicating that glucose is an effective and preferred carbon source for secondary metabolite synthesis by *Streptomyces* (Fguira et al., 2005). Also, Ouhdouch et al., (2001) stated that Bennett's medium was the best for antibiotic production among three media used in testing thirty-two strains of *Streptomyces*.

Table 2. Antimicrobial spectrum of the crude extracts of the isolates grown in Bennett's+Glucose and TSB media against several pathogens. The results represent the average mean zone diameters (in mm) of three replicates using the disk diffusion method

Isolates	Bennett's+Glucose					TSB				
	<i>E.</i>	<i>S.aure</i>	<i>S.pyogen</i>	<i>P.aerugino</i>	<i>C.albica</i>	<i>E.co</i>	<i>S.aure</i>	<i>S.pyogen</i>	<i>P.aerugino</i>	<i>C.albica</i>
AR4	-	-	14	-	-	-	-	-	-	-
AR6	-	-	-	-	-	-	-	-	-	-
AR9	-	18	18	-	-	-	-	-	-	-
AS28	-	10	-	-	-	-	-	-	-	-
AS36	-	-	-	-	-	-	-	-	-	-
BA12	8	12	16	-	5	-	-	-	-	-
BA14	10	18	12	-	5	-	-	-	-	-
BS29	12	18	20	-	7	-	-	-	-	-
BS32	-	10	-	-	-	-	-	-	-	-
CA12	-	-	-	-	-	-	5	-	-	-
CS41	-	30	24	-	-	-	16	12	-	-

Table 3. The results of the disk diffusion method for antibiotic susceptibility testing using standard antibiotics. The results represent the average mean zone diameters (in mm) of three replicates

Tested Organism	Imipenem	Netilmicin	Amoxicillin	Ofloxacin	Erythromycin	Amphotericin B
<i>S. aureus</i>	18	11	15	14	15	-
<i>S. pyogenes</i>	20	12	13	13	13	-
<i>C. albicans</i>	-	-	-	-	-	10
<i>E. coli</i>	15	13	11	14	-	-
<i>P. aeruginosa</i>	10	9	-	10	-	-

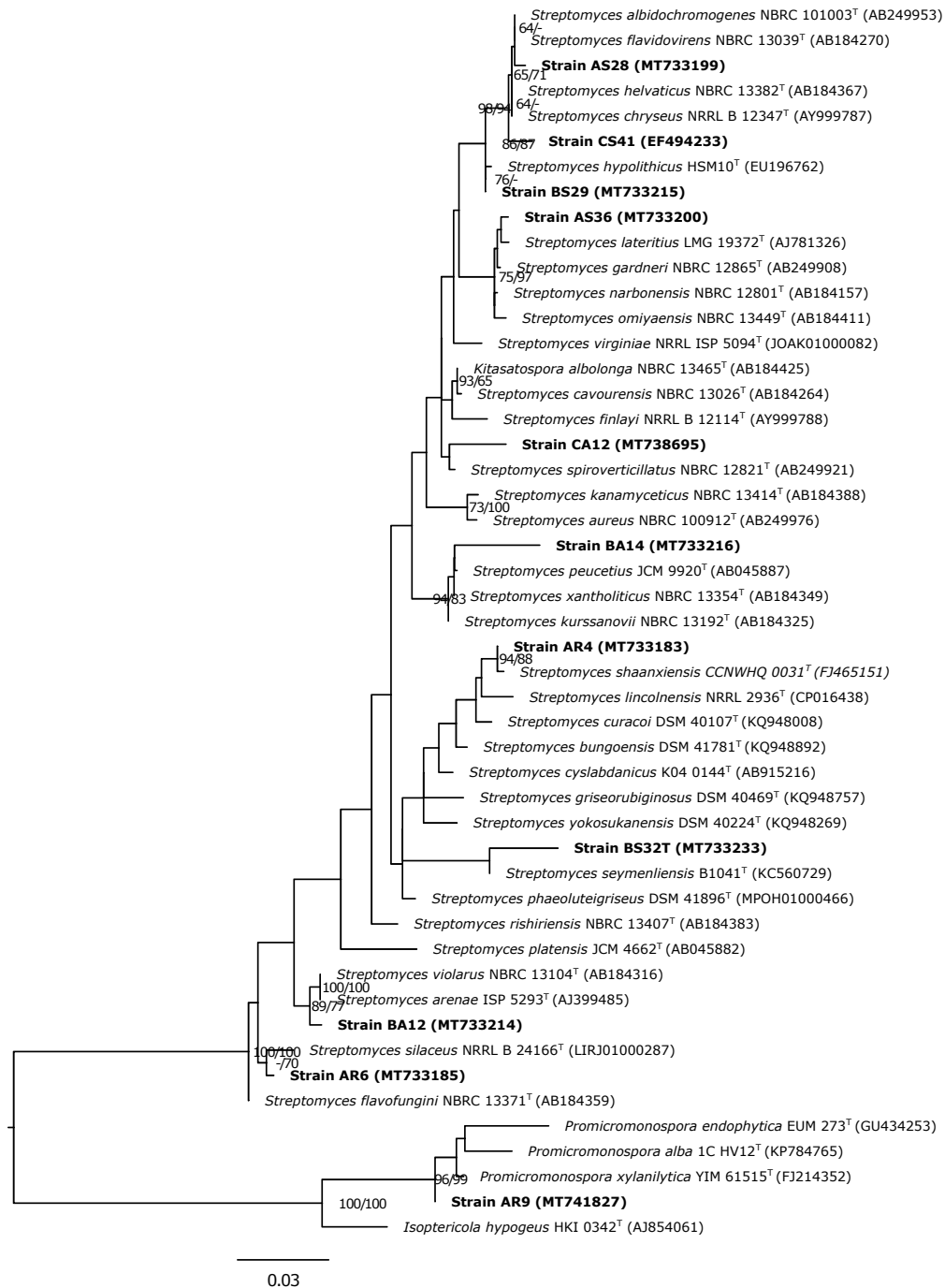


Figure 1. Phylogenetic tree based on the partial 16S rRNA gene analysis of the isolates. (The phylogenetic tree was constructed by the maximum likelihood method and rooted by midpoint-rooting. The branches are scaled in terms of the expected number of nucleotide substitutions per site. The numbers at nodes indicate the bootstrap support values when larger than 60% from maximum likelihood (left) and maximum parsimony (right) bootstrapping. GenBank accession numbers are given in brackets.)

Isolate AR9, our only isolate that does not belong to *Streptomyces* genus, was closely related to *Promicromonospora xylanilytica* (99.50%). There is a limited number of studies in the literature about this bacterium. It was a non-spore-forming and xylan-degrading bacterium that was isolated as endophytic actinomycete by Qin et al., (2012). In a recent study, the researchers discovered three new products from the *P. xylanilytica* YIM 61515. Their structures and cytotoxicity against five human cancer cell lines were elucidated (Wang et al., 2018). Therefore, chemical investigation of this isolate might open new windows to the discovery of new secondary metabolites.

The previous studies demonstrated the antimicrobial activity of secondary metabolites extracted from different *Streptomyces* strains against pathogens microorganisms. Kalyani et al., (2019) stated that the ethyl acetate extracts of *Streptomyces* sp. NLKPB45 isolated from mangrove soil samples showed antimicrobial activity against *Salmonella* sp., *S. aureus*, *E. coli*, and *B. subtilis* (Kalyani et al., 2019). Moreover, the researcher observed that the crude extract from the *Streptomyces* sp. possessed antimicrobial activity against *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Micrococcus luteus*, *S. aureus* with the MIC values ranging from 5 to 50 µg/mL (Riahi et al., 2019).

The culture-dependent/independent approaches may contribute to the discovery of new bioactive secondary metabolites from different microbial sources. Bioactivity screening is the first and most important step in secondary metabolite studies to highlight novel metabolites. Recent secondary metabolite screening studies have revealed that novel antibiotics (teixobactin, lugdunin and formicamycins etc.) are being discovered with these strategies from environmental microbial strains (Ling et al., 2015).

4. Conclusion

In this study, existing isolates were found to be involved in phylum Actinobacteria as a result of molecular diagnostic studies. Eight of them showed remarkable antimicrobial activity. The study about the effect of two different media on the production of bioactive metabolites indicated that using modified Bennett's medium provided higher antimicrobial activity when compared to TSB. Secondary metabolites of microbial origin are very important precursor compounds in studies conducted to investigate new drugs. The discovery of new compounds by screening bioactive metabolites in the *Streptomyces* genus with high secondary metabolite production potential is a possible situation.

Declarations

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