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Research Article

Taxonomic characterization and secondary metabolite production of newly isolated *Streptomyces* sp. MC12

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Abstract: An actinobacterium newly isolated from soil during a screening study was identified as *Streptomyces* sp. MC12 (GenBank accession number: PP757795) based on 16S rRNA analysis. For secondary metabolite production, fermentation was carried out in ISP 2 broth at 30°C, pH 7.3, for seven days under shaking conditions at 180 rpm. As a result of fermentation studies, the antagonistic effect of the crude extract, obtained through ethyl acetate extraction, against various microorganisms was determined. The MIC values of the extract against *Staphylococcus aureus* and *Escherichia coli* were 101.3 µg/mL and 153.6 µg/mL, respectively. It was also found to exhibit strong antifungal activity against *Penicillium* spp. *Streptomyces* sp. MC12, which displays both antifungal and antibacterial properties, is considered a potential secondary metabolite producer for future studies, particularly in pharmacology and the biocontrol of fungal pathogens.

1. INTRODUCTION

Microbial secondary metabolites include antibiotics, pigments, toxins, substances involved in symbiosis and competition, enzyme inhibitors, pheromones, agents affecting the immune system, receptor antagonists and agonists, pesticides, antitumor agents, and plant and animal growth promoters (Barka *et al*., 2016; Cuozzo *et al*., 2023). Among the drugs used in treatment, antibiotics are some of the most studied secondary metabolites. They are used to treat various infections in humans and animals. In addition to their therapeutic or protective purposes, antibiotics are also used in animal nutrition by incorporating them into daily feed to stimulate growth, resulting in increased weight. This practice has raised concerns regarding antimicrobial resistance. Moreover, antibiotics are used in food preservation and to promote plant growth in agriculture (Wassenaar, 2005; Salwan and Sharma, 2020; Nazari *et al*., 2023). Although many antibiotics are currently used in treatments, new screening studies are necessary due to the development of resistance in many pathogens. Among these resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for most hospital-acquired infections. Vancomycin-resistant *Enterococcus*, which complicates abdominal surgeries, and multi-drug-resistant *Mycobacterium tuberculosis* also pose significant challenges to scientists. Additionally, Gram-negative respiratory pathogens are increasingly developing resistance to

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antibiotics (Gauba and Rahman, 2023). The need for new antibiotics is growing rapidly as pathogens continue to develop resistance, rendering existing treatments inadequate or toxic (Demain, 1998). Recent studies on the discovery of bioactive metabolites often result in the re-isolation of well-known compounds, which can lead to significant losses in time, effort, and cost. This has prompted researchers to develop more effective discovery strategies (Anderson and Wellington, 2001; Jose *et al*., 2021).

Actinobacteria are undoubtedly the most important producers of pharmacologically active metabolites, especially antibiotics. Most actinobacteria, particularly *Streptomycetes*, are saprophytic, soil-dwelling organisms that spend much of their life cycle in semi-spore form, especially under nutrient-limited conditions. However, this phylum can adapt to a wide range of ecological environments: they are found in soil, fresh and saltwater, as well as in the air. They make up a significant part of the microbial population in alkaline soils rich in organic matter compared to other environments (Hazarika and Thakur, 2020; Selim *et al*., 2021). Additionally, they are found extensively in composts, straw, aquatic environments, and humid buildings.

Among actinobacteria, members of the *Streptomyces* genus produce some of the most important metabolites, such as antibiotics and enzymes. The *Streptomyces* genus includes a large group of organisms found in various natural habitats, producing a wide array of bioactive compounds, including antibiotics. More than half of the commercially and medically useful antibiotics derived from microbes are produced by actinobacteria, and 85% of these are from members of the *Streptomyces* genus (Watve *et al*., 2001; Hui *et al*., 2021).

Members of the *Streptomyces* genus are renowned for producing numerous secondary metabolites with antibacterial, antifungal, antiparasitic, antitumoral, and immunosuppressive biological activities (Chinnathambi *et al*., 2023). Moreover, it is estimated that less than 10% of *Streptomyces* bioactive compounds have been identified so far. Approximately 3,000 species of this genus have been described since the 1970s, including those with unconfirmed validity, making *Streptomyces* a rich source of new bioactive and commercially important compounds (Watve *et al*., 2001).

This study aims to identify the taxonomic characterization of the MC12 isolate based on morphological features and 16S rRNA sequence analysis, which produces high levels of antimicrobial metabolites during screening studies, and to assess the effectiveness of its secondary metabolites against different pathogens.

2. MATERIAL and METHODS

2.1. Isolation and Identification of the Isolate MC12

The soil sample was collected from the campus of Manisa Celal Bayar University (38°67'92.6"N, 27°30'17.1"E) in 2017, and actinobacteria were isolated using the soil dilution plate technique (Balagurunathan *et al*., 2020) on Yeast Malt Extract Agar (ISP 2) (Shirling and Gottlieb, 1966). This medium was supplemented with nystatin and streptomycin at a final concentration of 50 µg/mL to minimize fungal and bacterial contamination, respectively. The prepared petri dishes were incubated at 27-30°C for 7-14 days. After incubation, *Streptomyces*-like bacterial colonies (filamentous, rough, tough, leathery, chalky, and dusty) that grew on the medium were selected, purified on the same medium, and subjected to further experiments (Oskay, 2009; Sapkota *et al*., 2020).

The identification of the MC12 strain (with MC representing Muradiye Campus) was performed by partially sequencing 16S rRNA genes using oligonucleotide primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). DNA isolation of MC12 was carried out using the Genomic DNA Purification Kit (Promega, A1120) according to the manufacturer's protocol. The polymerase chain reaction (PCR) mixture (50 µL) contained 1 µL dNTPs (10 mM each), 4 µL $MgCl_2$ (25 mM), 1.5 µL forward primer (20 µM), 1.5 µL reverse primer (20 µM), 0.40 µL (5 U) Dream*Taq* DNA

polymerase (ThermoScientific, EP0702), 0.5 µL template DNA (approximately 50 ng), 5 µL 10× Dream*Taq* PCR buffer with $MgCl_2$, and 36.10 µL PCR-grade water. The sample was placed in a thermal cycler (Applied Biosystems Veriti Thermal Cycler, USA), and PCR was conducted with an initial denaturation at 96°C for 3 min, followed by 34 cycles of denaturation at 96°C for 45 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. The PCR product and molecular size marker (100 bp, Geneaid DL007) were subjected to electrophoresis in 1% agarose gel with TBE buffer, stained with safe DNA gel dye (Invitrogen), and examined using a gLite gel scanner. The single pure PCR product was commercially sequenced by GATC (Germany).

The sequence of MC12, along with related *Streptomyces* reference gene sequences obtained from GenBank (NCBI), was aligned using the MEGA XI (V 11.0.13) program (Tamura *et al*., 2021). The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) and Jukes-Cantor algorithms (Jukes and Cantor, 1969) with a bootstrap value of 1000 replicates (Felsenstein, 1985).

2.2. Secondary Metabolite Production by Fermentation

The seed culture of MC12 was prepared by inoculating a loopful of active culture into 250 mL Erlenmeyer flasks containing 50 mL of ISP2 broth (composition in g/L: yeast extract 4, malt extract 10, dextrose 4; pH 7.3) and incubated for three days at 28°C with shaking at 180 rpm. For fermentation, 2.5 mL (5%, v/v) of seed culture containing 1.0×10^6 spores/mL was inoculated into 50 mL of ISP2 broth. The fermentation was carried out at 28°C on a rotary incubator at 180 rpm for seven days. The fermentation broth was sampled at regular intervals (every 24 hours) and used in antimicrobial tests, and the process was stopped when optimal activity was observed. After determining the optimal production of secondary metabolites, the fermentation broth was centrifuged at 12,000 rpm at 4°C for 10 minutes to obtain the cell-free supernatant, which was then subjected to ethyl acetate extraction.

2.2.1. *Ethyl acetate extraction procedure of secondary metabolite*

Five different organic solvents—methanol, ethanol, ethyl acetate, hexane, and petroleum ether—were used for secondary metabolite extraction. Based on the antimicrobial test results of the obtained extracts, ethyl acetate was determined to be the most effective solvent and was subsequently used for all further extractions. Three hundred milliliters of cell-free supernatant, collected after centrifugation, was extracted twice with 300 mL of ethyl acetate at room temperature for 6 hours. After extraction, the ethyl acetate was evaporated using a rotary evaporator at 40°C, and the remaining extract was weighed. The extract was then dissolved in ethyl acetate at a concentration of 1 mg/mL and stored at -20°C for future experiments.

2.3. Determination of Antagonism

The primary antimicrobial activity of the identified *Streptomyces* sp. MC12 was assessed using the classical cross-streak method, following Oskay's protocol (Oskay, 2011) (data not shown). In secondary tests, the antimicrobial activity of the extracts obtained through fermentation was evaluated using the Well Diffusion method (Perez *et al.*, 1990) against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, and *Penicillium* spp. (isolated from lemon). The test microorganisms were obtained from the Department of Biology, Manisa Celal Bayar University. In this method, 20-60 µL of the extract, prepared at a concentration of 1 mg/mL, was transferred into pre-prepared wells (5 mm), and activity was determined based on inhibition zones. Experiments were generally carried out using a final concentration of 40 µL/well (40 µg/well).

For the determination of minimum inhibitory concentration (MIC), the microtiter broth dilution technique was used according to CLSI standards (CLSI, 2003 and 2006). Serial dilutions of the extract and reference antibiotics were prepared at concentrations of 50-500 µg/mL and 10-150 µg/mL, respectively. All other experimental conditions followed Oskay's method (Oskay, 2011). In secondary experiments, real MIC values were determined by

conducting tests at intermediate concentrations, adjusting the extract concentration as necessary. Parallel experiments were conducted with ethyl acetate as a negative control and reference antimicrobial agents (streptomycin and nystatin) as positive controls for comparison.

2.4. Ultraviolet Spectroscopy of Secondary Metabolite

Ultraviolet (UV) measurements of the *Streptomyces* sp. MC12 extract, dissolved in ethyl acetate, were taken at a wavelength of 200-800 nm using a UV/Visible spectrophotometer (Varian Cary 50 UV-Visible Spectrophotometer) to determine the maximum absorbance of the active substance(s).

2.5. Statistical Analysis

All experimental data were collected in triplicate, and the results were presented as the mean (M) ± standard deviation (SD). Statistical analysis was performed using Minitab® 19 software.

3. RESULTS

3.1. Identification of *Streptomyces* sp. MC12

As a result of morphological examinations, it was observed that MC12, isolated from soil, was a typical Gram-positive filamentous *Streptomyces*-like bacterium. Its colony formed extensively branched aerial and substrate mycelium in ISP 2 medium, with colors ranging from white to cream. The temperature range for growth was 20 to 37°C, with an optimal temperature of 28°C; however, no growth was observed at 4°C or 45°C. Since MC12 exhibited high activity in screening studies (Figure 1), it was identified by 16S rRNA sequencing.

Figure 1. Antagonistic activity of *Streptomyces* sp. MC12 against *Penicillium* spp. by cross-streak method. Medium: Potato Dextrose Agar.

First, the DNA of the strain was isolated, and PCR was performed under the reaction conditions described in the methods section. The agarose gel image of the PCR amplification product is shown in Figure 2a. According to the phylogenetic tree (Figure 2b), created by comparing the 16S rRNA sequence data (1,145 bp) with the sequences of closely related *Streptomyces* species, the strain showed 97% similarity to *Streptomyces enissocaesilis* NBRC 100763 and *Streptomyces rochei* NBRC 12908. However, due to the relatively low similarity rate, the strain, which produces high levels of secondary metabolites, was registered in GenBank as *Streptomyces* sp. MC12, with the accession number PP757795.

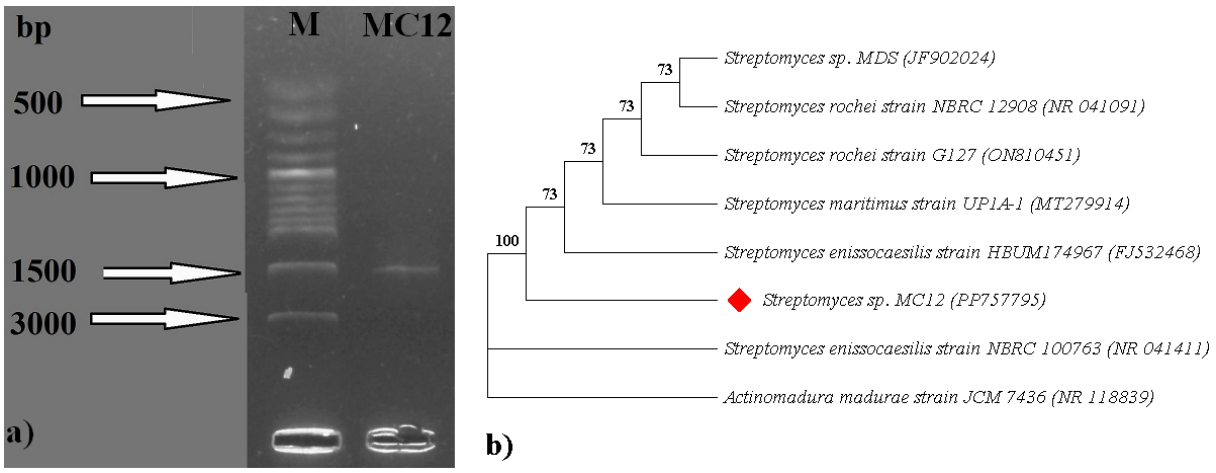

Figure 2. The agarose gel electrophoresis of the PCR product (a) and Comparative neighbour-joining phylogenetic tree based on 16S rRNA gene sequence of *Streptomyces* sp. MC12 with the sequences of closely related *Streptomyces* species (b). Bootstrap values calculated for 1000 replications are listed as percentages at branching points. Only bootstrap values >50% are shown.

3.2. Fermentation and Extraction of Secondary Metabolites

In this study, ISP 2 was selected as the fermentation medium, and only pH, temperature, and shaking speed were adjusted according to strain MC12 in preliminary experiments (data not shown). Secondary metabolite production by MC12 began at the 24th hour and reached its maximum on the 7th day. Since preliminary experiments determined that this isolate produced the highest yield of secondary metabolites on the 7th day, approximately 300 mL of cell-free culture supernatant was extracted twice with ethyl acetate in a 1:1 ratio. After evaporating the ethyl acetate from the extract, the remaining sample amount was approximately 5 mg. This extract was then dissolved to a concentration of 1 mg/mL and used in activity studies.

3.3. Antagonistic Effects of Secondary Metabolites against Different Microorganisms

The antagonistic effect of the ethyl acetate extract obtained at the end of fermentation was first tested using the Well Diffusion assay. As shown in Table 1, inhibition zones ranging from 10.6 to 20 mm were observed for the growth of the tested organisms at a concentration of 40 µg/well. The highest activity was observed against *E. coli* (20 mm), while the lowest activity was against *B. cereus* (10.6 mm). The activity against *Penicillium* sp. was also notable (14.6 mm).

Table 1. Well Diffusion assay and MIC results of the metabolite of *Streptomyces* sp. MC12 against test microorganisms.

Microorganisms[a]	Well diffusion assay (mm)[b]	MIC (µg/mL)	Streptomycin[c]	Nystatin
E. coli ATCC 25922	20.0±0.89[d]	153.6±2.87	20.3±1.3	NA
S. aureus ATCC 25923	18.6±0.51	101.3±2.73	8.5±0.44	NA
B. cereus ATCC 7064	10.6±1.03	ND	ND	NA
Penicillium sp.	14.6±1.03	ND	NA	27.0±0.89

[a] Bacteria tested in Mueller Hinton Agar, mould in PDA.
[b] Inhibition zone diameter in mm, not including well diameter (5 mm). The ethyl acetate extract concentration was 40 µg/well.
[c] MIC of the standard antimicrobial agents.
[d] Data presented as the M ± SD. ND - not determined, NA - not applicable.

Two microorganisms with high activity, *S. aureus* and *E.coli*, were selected and their MIC values were determined using the microdilution technique. MIC values against *S. aureus* and *E. coli* were determined as 101.3 and 153.6 µg/mL, respectively, and these values are comparable to MIC values of standard antimicrobial agents. The effect of ethyl acetate extract obtained from *Streptomyces* sp. MC12 at different concentrations against microorganisms is given in Figure 3.

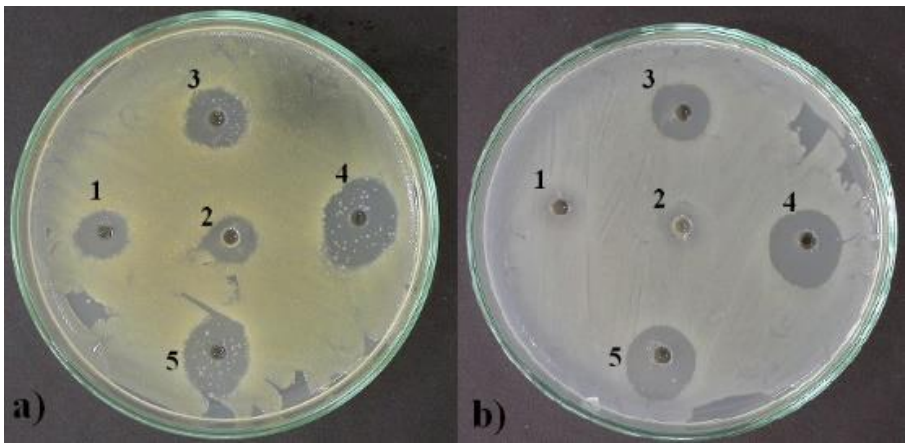

Figure 3. Antibacterial activity of the secondary metabolite of *Streptomyces* sp. MC12 against microorganisms; a) *S. aureus*, b) *E. coli*. Well diameter 5 mm, transferred volume 1) 20 µL, 2) 30 µL, 3) 40 µL, 4) 50 µL and 5) 60 µL/well at a concentration of 1000 µg/mL.

In the UV wavelength scanning of the ethyl acetate extract, the highest absorbance of the metabolite was observed between the 220-275 nm ranges (Figure 4). The UV absorbance value of the standard antibiotic streptomycin was in the range of 205-275 and was similar to the metabolite of *Streptomyces* sp. MC12.

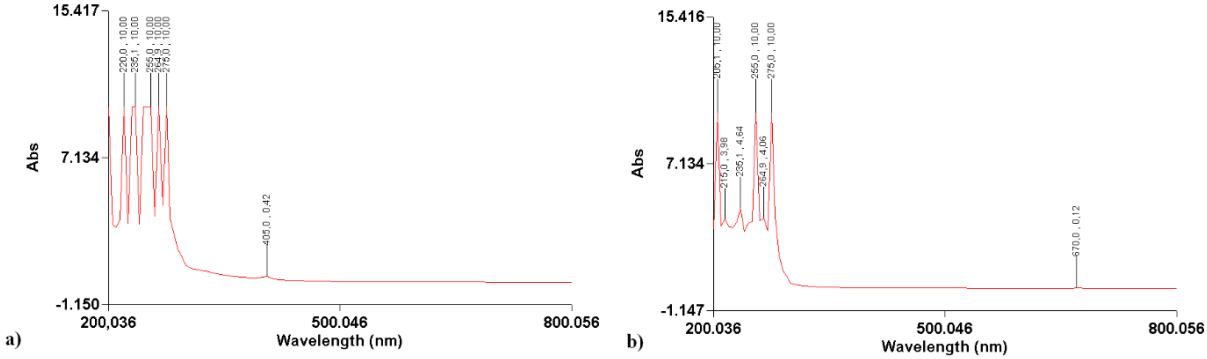

Figure 4. UV measurement at a wavelength of 200-800 nm; a) the secondary metabolites of *Streptomyces* sp. MC12 (30 µg /mL), b) standard antibiotic streptomycin (30 µg/mL).

4. DISCUSSION and CONCLUSION

In the current study, isolate MC12, which exhibited high inhibitory activity against certain pathogens during bioactive screening experiments, was characterized, and its secondary metabolite production was carried out through fermentation. Phylogenetic analysis of the 16S rRNA sequence, compared with known actinobacteria, placed MC12 within the genus *Streptomyces*. It showed 97% similarity to *Streptomyces enissocaesilis* NBRC 100763 and *Streptomyces rochei* NBRC 12908. However, due to the relatively low similarity for species identification, it was classified as *Streptomyces* sp. MC12 (PP757795) at the genus level. In numerous screening studies, secondary metabolite producers are generally identified as members of the *Streptomyces* genus.

ISP 2 is widely used and considered an ideal medium to produce secondary metabolites by fermentation of *Streptomyces* and related bacteria (Marzoug *et al*., 2023). Therefore, ISP 2, which proved to be suitable, was chosen for the fermentation of *Streptomyces* sp. MC12. Methanol, ethanol, and especially ethyl acetate are frequently used for metabolite extraction from fermentation supernatants (Al Farraj *et al*., 2020). In this research, ethyl acetate was selected as the appropriate organic solvent based on preliminary studies. The extract obtained (5 mg) was tested for activity against two Gram-positive bacteria (*S. aureus* and *B. cereus*), one

Gram-negative bacterium (*E. coli*), and one fungus (*Penicillium* spp.) from the culture collection of the Biology Department. The antibacterial activity varied with the concentration: *Streptomyces* sp. MC12 showed inhibition zones of 20 mm against *E. coli*, 18.6 mm against *S. aureus*, and 10.6 mm against *B. cereus*, using the Well Diffusion assay at a concentration of 40 µg/well. Almuhayawi *et al*. (2021) recorded the antibacterial activity of the Act19 isolate from desert habitat identified as *Streptomyces* sp. (MW240533) against *S. aureus*, *E. coli*, and *B. cereus* as 26 mm, 24 mm, and 28 mm, respectively. In another study, *Streptomyces albidoflavus* H12 showed strong activity against *Pseudomonas syringae*, *Pseudomonas corrugata*, and *Pectobacterium carotovorum* subsp. *carotovorum* (Djebaili *et al*., 2021). The isolate B3d, identified as *Streptomyces thermolilacinus*, demonstrated broad-spectrum antagonistic activity against multidrug-resistant bacterial pathogens such as *S. aureus, Salmonella* Typhi, *E. coli, Staphylococcus haemolyticus*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Talpur *et al*., 2020). *Streptomyces* sp. MC12 exhibited the highest activity against *S. aureus* and *E. coli*, with MIC values of 101.3 µg/mL and 153.6 µg/mL, respectively. These comparative results suggest that the secondary metabolites obtained from *Streptomyces* sp. MC12 may have a broad spectrum of antibiotic activity. For instance, *Streptomyces* sp. Kz-24 (KY000533) exhibited a MIC concentration of 0.024 µg/mL against methicillin-resistant *Staphylococcus aureus* ATCC 43300 and *Candida albicans* MTCC 227 (Sharma and Thakur, 2020). The findings of this research can be compared with other studies, where various *Streptomyces* species have been documented to possess significant antagonistic activity against fungal and bacterial pathogens (Sapkota *et al*., 2020; Chakraborty *et al*., 2022; Meena *et al*., 2023; Mirsonbol *et al*., 2023; Salehghamari *et al*., 2023).

Additionally, recent local research in Türkiye has focused on the production of secondary metabolites from actinobacteria, especially *Streptomyces*. Notable studies include those on actinobacteria isolated from various habitats, such as Karst Caves in the Eastern Black Sea Region (Tüfekci *et al*., 2023), Aras River (Seçkin *et al*., 2023), Kula Geopark (Bayraktar and Işık, 2024), Marmara and Avşa Islands (Topkara and Işık, 2023), symptomatic potatoes (Karagoz *et al*., 2024), lichen and orchid tissues (Ateş and Ay, 2023), legume nodules (Ay, 2020), and plant rhizosphere (Kum and İnce, 2021).

UV wavelength scanning of antibiotics provides valuable information about the active substances present in bacterial metabolites (Peris-Vicente *et al*., 2022). The UV absorbance value of the metabolite of *Streptomyces* sp. MC12 was in the 220-275 nm range and was similar to the absorbance peak of streptomycin (205-275 nm) (Figure 4). Most *Streptomyces*-derived antibiotics exhibit the highest absorbance values in the wavelength range of 200-400 nm. In a study, the UV peaks of cell-free filtrates from *Streptomyces* sp. HU2014 were found in the 291-352 nm range (Zhu *et al*., 2023).

Secondary metabolites are not essential for primary metabolic processes and growth in cells. Actinobacteria begin producing secondary metabolites in their natural environments, especially in soil, alongside morphological changes (Mehling *et al*., 1995; Mazumdar *et al*., 2023). Of over 23,000 known microbial secondary metabolites, 42% are produced by actinobacteria, 42% by fungi, and 16% by other bacteria. Approximately 45-55% of the 10,000 known antibiotics are produced by members of the *Streptomyces* genus (Watve *et al*., 2001; Hazarika and Thakur, 2020). The production mechanism of secondary metabolites is not fully understood but is thought to be related to sporulation (Barka *et al*., 2016; Zhu *et al*., 2023).

In the past 40 years, at least 15 million organisms have been isolated, and approximately ten thousand different antimicrobial substances have been identified in the search for new antibiotics. Each year, many of these are characterized, and 200-300 new ones are discovered. These studies have primarily focused on fungi, actinobacteria, and members of the *Bacillus* genus, which are known to be significant antibiotic producers. The genus *Streptomyces* is reported to be a major producer of bioactive secondary metabolites against various bacterial and fungal pathogens (Donald *et al*., 2023).

According to the current results, *Streptomyces* sp. MC12 exhibits broad-spectrum antagonistic activity against several microorganisms and could serve as a candidate for novel secondary metabolite discovery. Further studies should determine its species level, purify the bioactive metabolite, establish its structure, and conduct toxicity tests before it can be considered for therapeutic use.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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