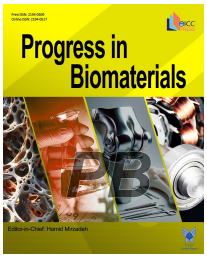

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Bioactive Metabolites from *Streptomyces mutabilis* PQ668646: Purification, Characterization, and Antibacterial and Antibiofilm Activities Against MRSA with Anticancer Potential

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Original Research

Abstract:

The escalating prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) underscores the critical need for novel antimicrobial agents capable of circumventing established resistance mechanisms. Actinomycetes, particularly *Streptomyces* species, remain one of the most prolific sources of clinically relevant natural antibiotics and represent a promising reservoir for new therapeutic compounds. In this study, we evaluated the antimicrobial and antibiofilm potential of actinomycete-derived metabolites against clinical MRSA isolates to identify candidates with translational potential for managing persistent and device-associated infections. A total of 165 *Staphylococcus* isolates were recovered from diverse clinical specimens and confirmed phenotypically as MRSA through standard microbiological and antimicrobial susceptibility assays. Ten actinomycete strains were isolated from soil samples, among which isolate no. 2 demonstrated the strongest anti-MRSA activity. Molecular analysis revealed a 1205 bp 16S rRNA gene sequence showing 100% identity to *Streptomyces mutabilis*. Phylogenetic analysis using the neighbor-joining method confirmed its close clustering with *S. mutabilis* strain PQ668646 with maximal bootstrap support. Bioactive metabolites extracted from *S. mutabilis* exhibited potent antibacterial activity across MRSA isolates, with a minimum inhibitory concentration (MIC) of 10.0 µg/mL and a minimum bactericidal concentration (MBC) of 20.0 µg/mL. Significant antibiofilm inhibition was observed ($p < 0.0001$), highlighting their potential to prevent biofilm-associated device infections. Cytotoxicity assays against PC-3 prostate cancer cells indicated additional anticancer activity ($IC_{50} + 73.4 \pm 0.19$ µg/mL). GC–MS profiling identified 30 metabolites, dominated by 9-octadecenamide (Z-), 1-docosene, and 9-hexacosene. HPLC confirmed 12 phenolic and flavonoid compounds, with syringic, ellagic, ferulic acid, and quercetin as major constituents. Collectively, these findings position *Streptomyces mutabilis* metabolites as promising candidates for developing novel antimicrobial and antibiofilm therapeutics against MRSA.

Keywords:

MRSA; Antibiotic resistance; Biofilm; *Streptomyces*; Secondary metabolites; Antibacterial activity; Antibiofilm activity; Anticancer activity

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1. Introduction

Staphylococcus aureus is a Gram-positive, coagulase-positive coccoid bacterium recognized as one of the most significant pathogens in clinical settings, and approximately $20-40\%$ of the general population carries *S. aureus* in the nasal mucosa as part of the normal human microbiota (El-Sherbiny et al., 2022). Moreover, when skin or mucosal barriers are compromised due to chronic dermatological conditions, injuries, or surgical interventions, *S. aureus* can invade deeper tissues or enter the bloodstream, with individuals having invasive medical devices or compromised immune systems at heightened infection risk. However,

the emergence of antibiotic-resistant bacteria represents a critical public health challenge, as MRSA, first identified in 1961, has become a leading cause of both nosocomial and community-acquired infections contributing to substantial morbidity and mortality worldwide (Becker et al., 2017). Therefore, understanding the molecular mechanisms underlying MRSA resistance is essential for addressing this global health threat. MRSA infections are characterized by waves of dominant strains rather than a single pandemic lineage, with notable emerging strains including healthcare-associated MRSA (HA-MRSA) in North America and Europe, community-associated MRSA (CA-MRSA) in North America, and livestock-associated MRSA strains such as ST398 and ST93 in Australia (Lee et al., 2018). Methicillin resistance is mediated by the mecA gene, carried on the mobile genetic element staphylococcal cassette chromosome mec (SCCmec), which encodes penicillin-binding protein 2a (PBP2a) that has reduced affinity for β-lactam antibiotics, conferring resistance across this entire antibiotic class (Turner et al., 2019). According to the World Health Organization, the aggregated prevalence of MRSA is 22.27% in the Americas, 16.57% in the Western Pacific, 10.93% in Europe, 8.55% in the Eastern Mediterranean, and 9.04% in Africa (Hasanpour et al., 2023).

Furthermore, biofilm formation further complicates MRSA infections, as the extracellular polymeric matrix restricts antibiotic penetration and facilitates survival of multidrug-resistant bacterial populations, with biofilm-associated bacteria demonstrating $10-1000$ times greater resistance to antimicrobial agents compared to planktonic bacteria. Biofilms allow bacteria to endure harsh environmental conditions, including fluctuations in pH, temperature, oxygen levels, and antimicrobial exposure, contributing to persistent and severe infections particularly on host tissues and medical devices (Balasubramanian et al., 2017; Asgeirsson et al., 2018; Verderosa et al., 2019; Kwiecinski and Horswill, 2020). The National Institutes of Health estimates that approximately 80% of recurrent microbial infections are linked to biofilm-forming bacteria (Kwiecinski and Horswill, 2020). The rapid emergence and global spread of antibiotic-resistant pathogens pose a major threat to public health, while the clinical pipeline for new antimicrobial agents has dramatically slowed in recent decades. This widening gap between escalating resistance and diminishing antibiotic innovation underscores the urgent need to discover and develop novel therapeutic compounds (Gavriilidou et al., 2022; Kalaba et al., 2024a).

Methicillin-resistant *Staphylococcus aureus* infections account for significant morbidity and mortality worldwide, and actinomycetes-particularly *Streptomyces* species- are recognized as the most prolific producers of bioactive secondary metabolites, synthesizing more than two-thirds of antibiotics currently used in clinical medicine (Kalaba et al., 2024b). Moreover, several frontline anti-MRSA drugs, such as vancomycin, teicoplanin, and daptomycin, originate from *Streptomyces*, while these specialized biomolecules display wide-ranging biological properties including antimicrobial, anticancer, immunosuppressant, and antiparasitic activities (O'Connor, 2015; Brown and Wright, 2016; Newman and Cragg, 2020; Atanasov et al., 2021). Therefore, this study explores *Streptomyces*-derived secondary metabolites as alternative sources of potent, structurally diverse antimicrobial compounds specifically targeting biofilm-mediated MRSA resistance. Microbial secondary metabolites represent an invaluable reservoir of natural products with exceptional chemical complexity and bioactivity, serving ecological functions including competition, defense, and signaling. *Streptomyces* species exhibit exceptional biosynthetic capacity, producing diverse antimicrobial classes including macrolides, β-lactams, polyenes, aminoglycosides, tetracyclines, polyketides, non-ribosomal peptides, and polyethers, underscoring the genus's central role in combating multidrug-resistant pathogens through novel therapeutic discovery (Kalaba et al., 2024b). Additionally, *Streptomyces* species possess large, GC-rich genomes densely populated with biosynthetic gene clusters that encode complex secondary metabolites through polyketide synthase, non-ribosomal peptide synthetase, hybrid, terpene, and ribosomally synthesized pathways, and modern approaches including elicitation, co-culture, CRISPR-based activation, and heterologous expression are increasingly used to awaken silent pathways for discovering novel anti-MRSA compounds (Atanasov et al., 2021). Moreover, advances in genome mining have revealed enormous untapped chemical diversity within the *Streptomyces* pan-genome, reinforcing their continued relevance as one of the most promising microbial resources for discovering next-generation antibiotics capable of addressing the escalating MRSA crisis (Newman and Cragg, 2020; Atanasov et al., 2021). However, previous research has predominantly centered on traditional antibiotic agents rather than comprehensive evaluations of multifunctional bioactive metabolites that could provide alternative therapeutic approaches against antimicrobial resistance. Therefore, this study explores the therapeutic potential of *S. mutabilis* as a source of multifunctional bioactive metabolites by integrating comprehensive evaluations of antimicrobial, antibiofilm, and anticancer activities associated with metabolites extracted from a newly identified *S. mutabilis* soil isolate, with the objective of inhibiting clinically relevant MRSA strains and contributing to the development of alternative therapeutic candidates capable of mitigating the growing threat of antimicrobial resistance.

2. Material and methods

2.1 MRSA isolates

2.1.1 Collection samples

Various clinical specimens used in this study were collected at the National Cancer Institute (NCI), Cairo University, Egypt, between May 2024 and September 2024, in full compliance with authorized national protocols and regulatory guidelines (IRB number: IRB-00004025; approval number: CB2410-102-083-192, 2024). The collected samples-including sputum, abscess exudates, aspirates, tissue biopsies, ear discharge, high vaginal swabs, throat swabs, pleural fluid, wound swabs, and skin lesion specimens-were placed in Amies transport medium and promptly transferred to the Bacteriology Laboratory, Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, for

comprehensive microbiological processing and analysis.

2.1.2 Cultivation and assessment of clinical samples

Assessment of the collected clinical samples was performed using standard microbiological techniques. Specimens were cultured on blood agar and mannitol salt agar plates (Oxoid, UK) and incubated at 37 °C for 24 hours. Distinct bacterial colonies were then isolated and identified using conventional morphological, biochemical methods, following the standard procedures described by El-Sherbiny et al. (2022).

2.2 Antibiotic susceptibility testing

Antimicrobial susceptibility of the identified isolates was assessed by the disc diffusion method using the following antibiotic discs: amikacin (AMK, 30 µg), cefaclor (CEC, 10 µg), ceftazidime (CAZ, 30 µg), erythromycin (E, 15 µg), amoxicillin (AXE, 25 µg), oxacillin (OX, 1 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), cefoxitin (FOX, 30 µg), ofloxacin (5 µg), ampicillin–sulbactam (SAM, 10/10 µg), ciprofloxacin (CIP, 5 µg), and doxycycline (DX, 30 µg). All discs were obtained from Oxoid Ltd. (England). Susceptibility testing and interpretation of inhibition zone diameters were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023).

2.3 Detection of biofilm formation

2.3.1 Congo red agar (CRA) method

Methicillin-resistant *Staphylococcus aureus* represents a significant clinical challenge in healthcare settings, and biofilm formation by MRSA isolates substantially increases their resistance to antimicrobial treatments and host immune responses. However, effective management of MRSA infections requires reliable screening methods to identify biofilm-forming strains and distinguish them from non-biofilm producers. Therefore, we employed qualitative screening for biofilm formation among MRSA isolates using Congo red agar (CRA) methodology. The CRA medium contained (g/L): brain heart infusion (37.0), sucrose (50.0), agar (10.0), and Congo red (0.8) (Sigma-Aldrich, Germany). Isolates were streaked onto CRA plates and incubated at 37 °C for 24 hours. This screening approach successfully differentiated biofilm-forming strains, which appeared as black, dry, crystalline colonies, from non-biofilm formers that produced red or pink colonies, providing a practical and reliable method for biofilm detection in MRSA isolates (Ahmad et al., 2022).

2.3.2 Tube method (TM)

Qualitative biofilm assessment methods require careful standardization to ensure reproducible and interpretable outcomes. Therefore, a modified tube-based qualitative assay, adapted from El-Sherbiny et al. (2023), was employed to evaluate biofilm formation among the tested bacterial isolates. Briefly, a loopful of each isolate was inoculated into 10 mL of tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37 °C for 24 h. Following incubation, the broth was decanted, and the tubes were gently rinsed with phosphate-buffered saline (PBS; pH 7.3), air-dried, and stained with 0.1% crystal violet. Excess stain was removed by washing with deionized water, and the tubes were left to dry in an inverted position. Biofilm formation was evaluated visually, with the presence of a stained film along the inner wall of the tube considered indicative of positive biofilm production. Biofilm strength was semi-quantitatively scored as follows: 1 = weak/none, 2 = moderate, and 3 = strong. All assays were performed in triplicate and independently repeated three times to ensure data reliability. This standardized and cost-effective approach supports consistent qualitative screening of biofilm formation and facilitates comparative analysis across bacterial isolates.

2.3.3 Microtiter plate assay (MPA)

Semi-quantitative biofilm assays using microtiter plates are widely regarded as the gold standard for assessing bacterial biofilm-forming ability due to their reproducibility, cost-effectiveness, and suitability for high-throughput analysis. To ensure consistency and comparability across isolates, we performed a standardized microtiter plate biofilm assay following established methodologies (Cotter et al., 2009; Siddique et al., 2020). Briefly, each bacterial isolate was cultured in tryptic soy broth (TSB; Oxoid, UK) supplemented with 1% glucose and incubated at 37 °C for 24 h. Cultures were then diluted and inoculated into sterile 96-well flat-bottom microtiter plates. After incubation, non-adherent cells were removed by gentle washing with phosphate-buffered saline (PBS; pH 7.2). The remaining surface-attached biofilms were air-dried, fixed, and stained with 0.1% crystal violet (Oxford, UK). Excess stain was removed, and the bound dye was solubilized in 95% ethanol. Biofilm biomass was quantified by measuring optical density (OD) at 492 nm using a microplate reader. All assays were conducted in triplicate to ensure statistical robustness. Biofilm-forming capacity was classified according to the established criteria presented in Table 1, allowing standardized comparison among isolates. This systematic and validated approach enables reliable quantification and categorization of biofilm formation, supporting comprehen-

Table 1. Classification of bacteria based on biofilm formation capacity.

Biofilm Class	Results
$OD > 4 \times ODc$	Strong biofilm
$2 \times ODc < OD \leq 4 \times ODc$	Medium biofilm
$ODc < OD \leq 2 \times ODc$	Poor biofilm
$OD \leq ODc$	Negative biofilm

sive evaluation of this critical virulence attribute in bacterial pathogenesis research.

2.4 Actinomycetes isolates

2.4.1 Isolation of actinomycetes isolates and antibacterial activity

Antibiotic-resistant bacteria, particularly MRSA, pose significant threats to global health, and actinomycetes from soil environments have demonstrated considerable potential as sources of novel antimicrobial compounds. However, systematic evaluation of actinomycetes isolated from Egyptian soil for anti-MRSA activity remains underexplored, limiting our understanding of local microbial resources for combating antibiotic resistance. Therefore, we conducted a comprehensive study to isolate soil actinomycetes and evaluate their antibacterial efficacy against MRSA isolates. Soil samples were collected during August and September 2024 from an area adjacent to the Faculty of Science Garden, Al-Azhar University, Cairo, Egypt. Samples were placed in sterile, airtight polyethylene bags and transported to the laboratory for actinomycetes isolation using ISP4 agar medium. The ISP4 medium composition per liter included: 2.0 g KNO_3, 20.0 g starch, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 0.5 g NaCl, 3.0 g $CaCO_3$, and 0.01 g $FeSO_4 \cdot 7H_2O$, supplemented with 20 g agar in distilled water to a final volume of 1 L, with pH adjusted to 7.0 ± 0.2. For seed culture preparation, three 9-mm discs from seven-day-old cultures were inoculated into 100 mL of starch nitrate broth (pH 7.2 ± 0.2) in 250-mL Erlenmeyer flasks. Cultures were incubated at 30 °C for 48 hours on a rotary shaker at 150 rpm. Following incubation, cultures were filtered through 0.45-µm Whatman filter paper and centrifuged at 10,000 × g for 15 minutes to obtain cell-free filtrate (CFF). Antibacterial activity of each CFF was evaluated against MRSA isolates using the agar well diffusion method. Wells of 6 mm diameter were prepared using a sterile cork borer, and 100 µL of each filtrate was dispensed into individual wells. Cefoxitin served as positive control. Plates were pre-incubated at 4 °C for 2 hours to facilitate diffusion, followed by incubation at 37 °C for 24 hours. Inhibition zone diameters were measured in millimeters according to Clinical and Laboratory Standards Institute guidelines (CLSI) guidelines (CLSI, 2023).

2.4.2 Identification of the most potent streptomyces isolate

Pure isolates were preliminarily characterized based on their morphological, physiological, and biochemical properties, followed by molecular identification (Rammali et al., 2022). The most active isolate was subjected to partial 16S rDNA sequencing. Genomic DNA was extracted from cultures harvested during the late exponential phase using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA; K0791), following the manufacturer's protocol. PCR amplification of the 16S rDNA gene was performed using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′) on a BIO-RAD T100 thermocycler (BIO-RAD Laboratories, Hercules, CA, USA). The PCR conditions included an initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 54 °C for 30 seconds, extension at 72 °C for 2 minutes, and a final extension at 72 °C for 5 minutes. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA; K0701). The resulting 16S rDNA sequence was analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine similarity with reference sequences in the GenBank database. Phylogenetic analysis was performed using MEGA version 6.0, which supports evolutionary timetree reconstruction and is available at https://www.megasoftware.net/reltime Pure isolates were preliminarily characterized based on their morphological, physiological, and biochemical properties, followed by molecular identification (Liu et al., 2009; Kumar et al., 2018).

2.4.3 Extraction and purification of bioactive metabolites

Streptomyces species are renowned producers of bioactive secondary metabolites with significant pharmaceutical and biotechnological applications, and standardized fermentation and extraction protocols are essential for isolating these valuable compounds from complex culture broths. However, the separation and purification of specific bioactive metabolites from *Streptomyces* fermentation mixtures remains challenging due to the complexity of the chemical matrix and the need for efficient chromatographic resolution. Therefore, we developed a systematic approach combining optimized fermentation conditions with sequential extraction and preliminary chromatographic separation to isolate bioactive compounds from the most potent *Streptomyces* isolate. The selected isolate was inoculated at 10% (v/v) into a 1 L Erlenmeyer flask containing 250 mL of starch nitrate broth and incubated at 30 °C for seven days with continuous agitation at 150 rpm. Following fermentation, the broth culture was filtered through a Whatman 0.45 µm membrane filter and centrifuged at 10,000 × g for 30 minutes at 4 °C to obtain cell-free filtrate. Bioactive metabolites were extracted twice with ethyl acetate (1:1, v/v) using a separatory funnel, and the combined organic layers were concentrated under reduced pressure at 45 °C using a rotary evaporator. For preliminary chromatographic separation, the concentrated crude extract was applied as a narrow band onto a 20 × 20 cm silica gel TLC plate using diethyl ether: n-hexane (7:3, v/v) as the mobile phase. After development to 16 cm from the origin, examination under UV light at 254 nm revealed a prominent brown, fluorescent band with an Rf value of 0.72, which was subsequently scraped off, eluted with methanol, and dried for further analysis. This systematic approach successfully achieved preliminary separation of a distinct bioactive compound, demonstrating the effectiveness of the combined fermentation-extraction-chromatographic methodology for isolating *Streptomyces*-derived bioactive metabolites (El-Sherbiny et al., 2023).

2.4.4 Spectroscopic analysis of the bioactive metabolite

2.4.4.1. Gas chromatography-mass spectrometry (GC-MS)

Accurate characterization of purified metabolites is fundamental to biochemical research, and gas chromatogra-

phy–mass spectrometry has emerged as a gold standard technique for compound identification and structural elucidation. However, reliable metabolite identification requires precise analytical protocols and comprehensive spectral database comparisons to ensure reproducible results. Therefore, we employed a systematic GC-MS analytical approach following the established method of El-Sherbiny et al. (2023) to characterize purified metabolites through comprehensive mass spectral analysis. The purified metabolite was dissolved in spectroscopy-grade methanol and analyzed using a Trace GC1310–ISQ mass spectrometer equipped with a TG-5MS capillary column (30 m × 0.25 mm × 0.25 µm; Thermo Scientific, Austin, Texas, USA). The oven temperature program included an initial temperature of 50 °C, increased at 5 °C/min to 230 °C and held for 2 minutes, then increased to 290 °C and held for 2 minutes. Injector and MS transfer line temperatures were maintained at 250 °C and 260 °C, respectively, with helium serving as the carrier gas at a split ratio of 1:30. The mass spectrometer operated in electron ionization mode at 70 eV, with an ion source temperature of 200 °C and a scan range of $40-1000$ m/z. Component identification was achieved through systematic comparison of obtained mass spectra with both NIST 11 and WILEY 09 mass spectral libraries, ensuring comprehensive database coverage for reliable compound characterization. This standardized analytical protocol provides a robust framework for metabolite identification with high confidence through dual-database spectral matching.

2.4.4.2. HPLC Analysis

2.4.7.1.1. Sample preparation

A precisely weighed sample (100 mg) of the extracted purified metabolite was dissolved in 10 mL of 50% methanol, filtered through a 0.2 µm nylon membrane, and analyzed in triplicate.

2.4.7.1.2. HPLC apparatus and chromatographic conditions

Metabolite profiling represents a fundamental analytical approach in biochemical research, and high-performance liquid chromatography serves as a cornerstone technique for separating and identifying complex metabolite mixtures. However, achieving reliable metabolite separation and accurate detection requires precise optimization of chromatographic conditions and systematic documentation of analytical parameters. Therefore, we employed a comprehensive HPLC methodology using a Shimadzu HPLC system (SPD-10A, Kyoto, Japan) equipped with a SIL-10AD autoinjector, SPD-10AV UV–Vis detector ($\lambda = 280$ nm), DGU-10A degasser, and LC-10AD pump to analyze metabolite profiles. Separation was achieved using a Shim-pack CLC-ODS (C18) analytical column (2 cm × 4.6 mm, 5 µm) coupled to a C18 guard column. The mobile phase consisted of solvent A (1% acetic acid in water; H_2O: AA 94:6, pH 2.27) and solvent B (acetonitrile). The gradient elution program was optimized as follows: $0-15$ min, 15% B; $15-30$ min, 45% B; $30-45$ min, 100% B. The flow rate was maintained at 1 mL/min at ambient temperature. Chromatographic peaks were systematically recorded, and retention times were documented for each detected compound, providing a robust analytical framework for metabolite identification and quantification (Khalid Mu et al., 2023).

2.4.5 Determination of MIC and MBC of purified bioactive metabolite

The MIC and MBC of the purified bioactive compound against MRSA isolates were determined using the standard broth microdilution assay in sterile 96-well microtiter plates, with gentamicin included as a positive control. Briefly, 100 µL of Mueller–Hinton broth (MHB) was dispensed into each well, followed by two-fold serial dilutions of the test compound. Gentamicin was tested at concentrations ranging from 0.1 to 15.0 µg/mL, while the bioactive compound was evaluated across a concentration range of 0.0 to 50.0 µg/mL. Subsequently, 10 µL of a standardized MRSA suspension (approximately 10^6 CFU/mL) was added to each well. The plates were incubated at 37 °C for 24 h, and bacterial growth was assessed spectrophotometrically at 630 nm. The MIC was defined as the lowest concentration showing complete inhibition of visible growth, whereas the MBC was determined as the lowest concentration causing $\geq 99.9\%$ reduction in viable bacterial count, in accordance with CLSI guidelines (CLSI, 2023).

2.4.6 Antibiofilm activity

To evaluate the antibiofilm activity of the active metabolite against MRSA strains, sub-inhibitory concentrations (1/8, 1/4, and 1/2 MIC) were applied based on the previously determined MIC values. Biofilm formation was quantified using the crystal violet assay according to standard protocols. Untreated wells containing medium alone or medium with bacterial inoculum served as negative and positive controls, respectively. This methodology allows the assessment of the active metabolite's potential to disrupt or inhibit MRSA biofilm development at sub-therapeutic concentrations, offering a promising strategy for combating biofilm-associated MRSA infections.

2.4.7 Cytotoxicity and anticancer activity

Natural products represent a valuable source of bioactive compounds for cancer therapy development, and systematic screening of purified metabolites against multiple cancer cell types provides essential data for identifying promising anticancer candidates. However, comprehensive cytotoxicity evaluation of newly purified bioactive metabolites against both normal and diverse cancer cell lines remains limited, hindering the identification of compounds with selective anticancer activity. Therefore, we evaluated the cytotoxicity and anticancer activity of a purified bioactive metabolite in vitro against the human normal cell line HFB-4 and cancer cell lines HepG2, MCF-7, and PC-3, following established protocols. Cell viability and proliferation were assessed using the MTT assay with serial metabolite concentrations ($0-1000$ µg/mL) and 24-hour incubation periods. After incubation, cells were rinsed with fresh medium or cold PBS and treated with MTT solution (0.5 mg/mL) for $2-5$ hours. Following MTT reagent removal, 200 µL of DMSO was added to solubilize formazan crystals, and absorbance was measured at 570 nm. Percentages of cell viability and cytotoxicity were calculated using standard

formulas (Sharaf et al., 2021).

$$\%\text{Cell viability} = \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \times 100$$

$$\%\text{Cell death} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

2.5 Statistical analysis

All experiments were conducted in triplicate, and data are presented as mean ± standard deviation. Statistical comparisons between treated and control groups were performed using two-way ANOVA in GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Result and discussion

3.1 Isolation, identification and antibiotic profile of MRSA isolates

Staphylococcus aureus represents one of the most clinically significant bacterial pathogens, causing a wide spectrum of infections ranging from superficial skin conditions to life-threatening systemic diseases, and accurate identification of this organism is fundamental to appropriate clinical management and infection control measures. However, reliable phenotypic characterization of *S. aureus* requires comprehensive evaluation of multiple morphological, physiological, and biochemical characteristics to ensure diagnostic accuracy and prevent misidentification (El-Sherbiny et al., 2022). Therefore, we conducted systematic phenotypic analysis of bacterial isolates recovered from clinical samples to establish definitive *S. aureus* identification through multiple complementary testing approaches. A total of 165 bacterial isolates were recovered from the collected clinical samples and were identified as *S. aureus* based on their morphological, physiological, and biochemical characteristics. All isolates appeared as Gram-positive cocci arranged in clusters, produced golden-yellow colonies on nutrient agar, and exhibited β-hemolysis on blood agar. They also tested positive for catalase, coagulase, and mannitol fermentation, consistent with previously reported phenotypic traits of *S. aureus*. This comprehensive phenotypic characterization approach successfully identified all 165 isolates as *S. aureus* through consistent demonstration of the organism's characteristic features across multiple testing parameters (Becker et al., 2017; Lee et al., 2018; Hasanpour et al., 2023; El-Sherbiny et al., 2022). Methicillin-resistant *Staphylococcus aureus* represents a major clinical challenge in both hospital-acquired and community-associated infections, and accurate antibiotic susceptibility testing is essential for guiding effective treatment strategies. However, the emergence of multidrug resistance patterns in MRSA isolates threatens to limit therapeutic options and compromise patient outcomes. Therefore, we conducted comprehensive antibiotic susceptibility testing using 12 antibiotics representing different classes to characterize resistance patterns in clinical MRSA isolates. All isolates were confirmed as MRSA, showing resistance to both cefoxitin and oxacillin, with a high prevalence of multidrug resistance observed as most isolates demonstrated resistance to the majority of tested antibiotics. Among the evaluated antibiotics, amikacin and doxycycline exhibited the highest activity, with 161 (95.8%) and 147 (87.5%) isolates, respectively, remaining susceptible (figure 1). Both methicillin- and multidrug-resistant *S. aureus* strains were recovered from hospital-acquired and community-associated infections, indicating the widespread distribution of resistance patterns across different clinical settings (Becker et al., 2017; El-Sherbiny et al., 2022). These findings provide critical insights into current MRSA

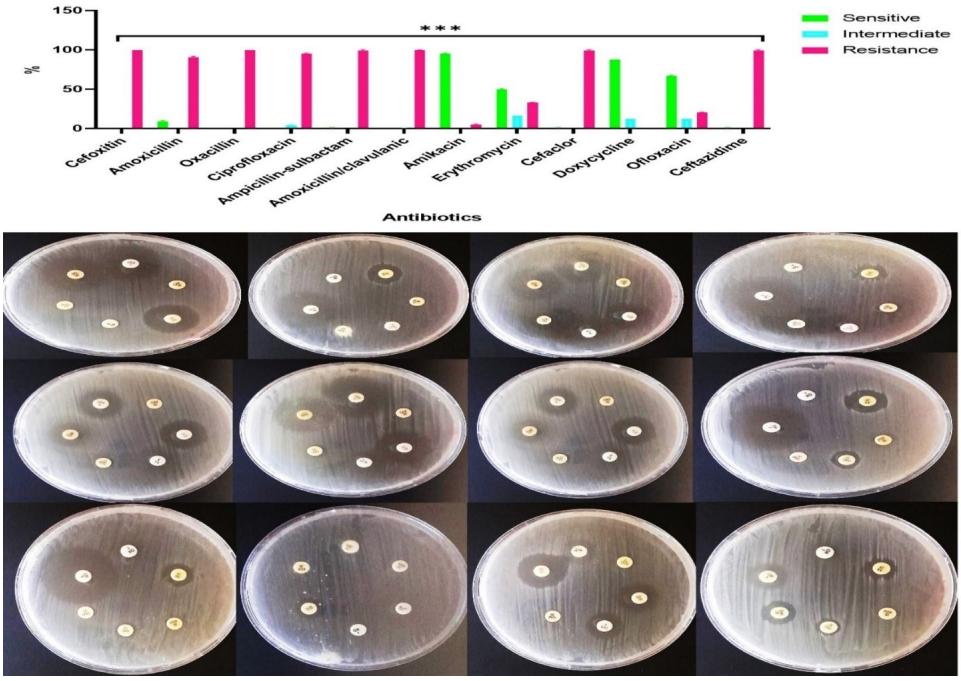


Figure 1. Antibiotics profile of MRSA isolates (**** $p < 0.0001$).

susceptibility patterns and identify amikacin and doxycycline as potentially effective therapeutic options against multidrug-resistant isolates. These findings align with a previous report by Vijayamohan and Nair (2014), who documented that 93.75% of MRSA isolates retained sensitivity to amikacin. They also emphasized that amikacin is more cost-effective and widely accessible than vancomycin or linezolid, supporting its use as a potential first-line treatment option for MRSA, particularly in resource-limited settings or where access to advanced antimicrobials is constrained.

3.2 Detection of biofilm formation by MRSA isolates

Biofilm formation by bacteria depends on both the availability of a suitable substrate and favorable environmental conditions, and surface roughness plays a critical role by providing a larger area for microbial attachment and offering physicochemical properties that promote colonization and subsequent biofilm maturation. However, the quantitative relationships between specific surface roughness parameters and biofilm development rates, as well as the underlying mechanisms governing these interactions, remain poorly understood across different bacterial species and environmental conditions. Therefore, systematic investigation of how surface topography influences microbial colonization patterns and biofilm architecture is essential to advance our understanding of bacterial adhesion processes and develop more effective strategies for controlling biofilm formation in both beneficial and detrimental applications. (Ghazi et al., 2017; Kaushik et al., 2024; Omar et al., 2024). To accurate assessment of biofilm-forming capability in MRSA isolates remains challenging due to variability between detection methods and the need for reliable quantitative approaches. Therefore, we applied three complementary techniques-Congo red agar, the tube method, and the microtiter plate assay-to comprehensively assess biofilm formation among MRSA isolates. All isolates demonstrated biofilm-forming capability across the different assays. Quantitative analysis using the microtiter plate assay revealed that 45 isolates (26.7%) were strong biofilm producers, 107 isolates (63.7%) exhibited moderate biofilm production, and 16 isolates (9.6%) showed weak biofilm formation (Table 2, Fig. 2 A-C). These findings demonstrate the universal biofilm-forming capacity of MRSA isolates while revealing significant variation in biofilm production strength, providing crucial insights for clinical management strategies and highlighting the importance of using multiple complementary methods for accurate biofilm assessment. These results are consistent with previous reports by Atshan and Shamsudin (2011), who confirmed universal biofilm formation among clinical MRSA isolates, and Deepak et al. (2024), who also documented high frequencies of biofilm

Table 2. Biofilm formation by MRSA isolates.

Categories of biofilm	Congo red	Tube method	Microplate assay
Strong	165 (100%)	50 (29.8%)	45 (26.7%)
Moderate	0 (0.0%)	101 (60.1%)	107 (63.7%)
Week	0 (0.0%)	17 (10.1%)	16 (9.6%)
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)

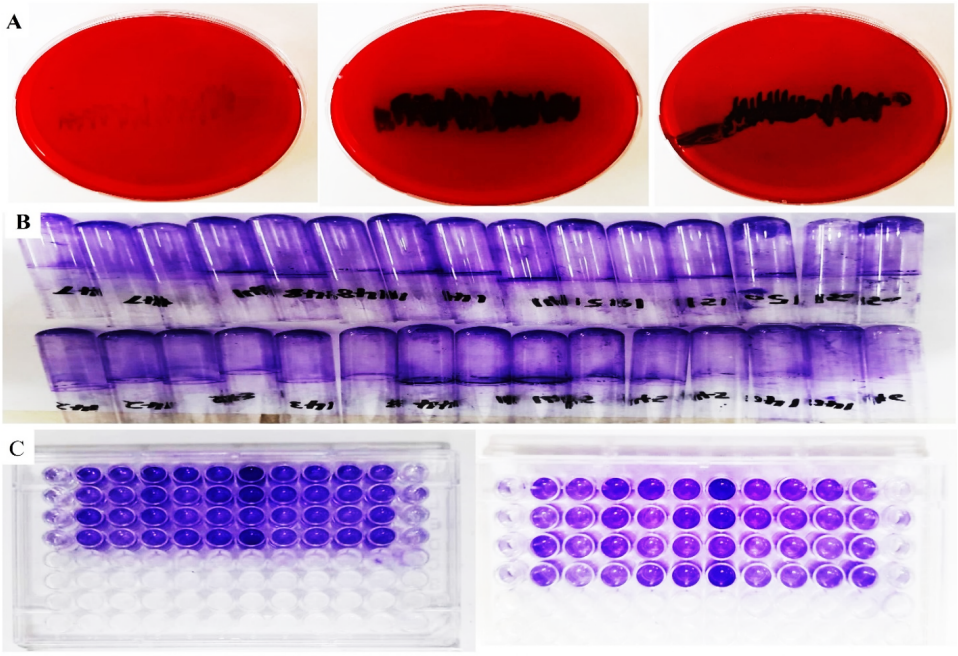


Figure 2. Detection of biofilm formation by MRSA isolates using different methods: (A) Congo red agar (CRA), (B) Tube methods (TM), and (C) Microtiter plate assay (MPA).

production in MRSA strains. *Staphylococcus aureus* is capable of forming biofilms on both biotic and abiotic surfaces, contributing significantly to its persistence and increased resistance to antimicrobial agents. Cells residing within biofilms display distinct phenotypic properties compared to their planktonic counterparts-such as enhanced antibiotic tolerance, altered growth behavior, morphological modifications, differential gene regulation, and changes in protein expression (Asgeirsson et al., 2018). MRSA continues to represent a major global health concern, frequently associated with chronic, recurrent, and difficult-to-treat infections. Biofilm formation further strengthens its ability to evade host immune responses and withstand therapeutic interventions, leading to increased morbidity and mortality across diverse patient populations Kaushik et al. (2024). Biofilm-associated MRSA infections span a wide clinical spectrum, from skin and soft tissue infections (SSTIs) to severe invasive diseases including bloodstream infections (BSIs), osteomyelitis, and infective endocarditis (IE) (Asgeirsson et al., 2018; Riche et al., 2023).

3.3 Isolation of anti-MRSA active actinomycete

Streptomyces species are widely recognized as exceptional producers of bioactive secondary metabolites, accounting for nearly 75% of clinically used antibiotics and serving as a prolific source of anti-MRSA agents, including vancomycin. Nevertheless, the rapid emergence and global spread of antibiotic-resistant pathogens-particularly MRSA-highlight an urgent need to discover and develop novel therapeutics capable of overcoming current resistance challenges Janardhan et al. (2014). Therefore, we isolated ten actinomycete isolates from garden soil collected at the Faculty of Science, Al-Azhar University, Cairo, Egypt, and screened them for antibacterial activity against MRSA isolates. The isolates were cultivated in starch nitrate medium and subsequently tested for their antimicrobial properties. Most of the actinomycete isolates exhibited significant antibacterial activity ($p < 0.0001$), with the exception of isolate number 10. Among the tested isolates, isolate number 2 demonstrated the most potent activity against MRSA as shown in figure 3. These findings reinforce the importance of actinomycetes-particularly *Streptomyces* spp.-as a valuable reservoir for discovering new antimicrobial agents with potent activity against clinically significant resistant pathogens such as MRSA. Our results align with previous studies, including those by Kemung et al. (2018), who reported that *Streptomyces* species have been the source of multiple anti-MRSA compounds, most notably vancomycin. Moreover, Hughes et al. (2010), demonstrated the continued therapeutic potential of this genus by isolating marinopyrroles with strong anti-MRSA activity from the marine-derived strain *Streptomyces* sp. CNQ. Collectively, these findings highlight *Streptomyces* as a persistent and promising target for bioprospecting efforts aimed at combating antimicrobial resistance.

3.4 Identification of the most potent actinomycete isolate

Actinomycetes represent a crucial source of bioactive compounds and industrial enzymes, and systematic phenotypic characterization using standardized media provides essential taxonomic and biotechnological insights for these microorganisms. However, comprehensive phenotypic evaluation of promising actinomycete isolates often remains incomplete, limiting our understanding of their growth characteristics and potential applications. Therefore, we conducted a systematic phenotypic characterization of the most promising actinomycete isolate (isolate no. 2) using International *Streptomyces* Project (ISP) media to evaluate its comprehensive growth characteristics. The isolate exhibited strong to moderate growth on ISP-3, ISP-4, ISP-5, and ISP-7, while demonstrating moderate growth on ISP-1, ISP-2, and ISP-6. Morphological analysis revealed that the aerial mycelium ranged from white to grey, whereas the substrate mycelium appeared beige to brown, with no production of diffusible pigments observed on any ISP medium. These standardized phenotypic characteristics provide essential baseline data for taxonomic classification and biotechnological potential assessment of this promising actinomycete isolate figure 4 A. Micromorphological examination on in-

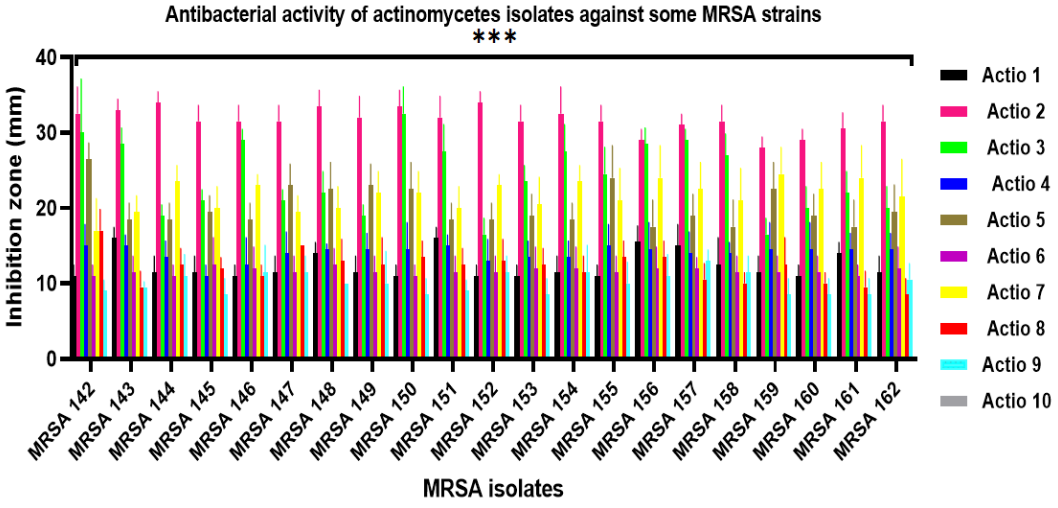


Figure 3. Antibacterial activity of actinomycetes isolates against some MRSA isolates (**** $p < 0.0001$).

organic salts-starch agar (ISP-4) revealed straight aerial hyphae that differentiated into smooth-surfaced spores. Scanning electron microscopy further confirmed the presence of spore chains composed of approximately 10-15 spores, each with an average diameter of ∼ 0.10 µm (figure 4 B). The cultural and microscopic characteristics were highly consistent with those described for *Streptomyces mutabilis*, which typically produces brown substrate mycelium, light to medium grey aerial mycelium, and short spore chains arranged in loops, hooks, or loose spirals, without formation of sporangia or other specialized reproductive structures (Toumatia et al., 2015). Molecular identification was performed by amplifying and sequencing the 16S rRNA gene. Electrophoresis confirmed a distinct amplicon of ∼ 1205 bp (figure 4 C). Phylogenetic analysis using the neighbor-joining method in MEGA X revealed that the isolate clustered tightly with reference sequences of *S. mutabilis*, with a scale bar representing 0.20 substitutions per nucleotide position (figure 4 D). Multiple sequence alignment confirmed 100% identity with *S. mutabilis*. The validated sequence was deposited in GenBank under accession number PQ668646 (https://www.ncbi.nlm.nih.gov/search/all/?term=PQ668646). These findings are in agreement with Belghit et al. (2016), who confirmed *S. mutabilis* as a soil-derived species through combined phenotypic and 16S rRNA gene analyses.

3.5 Purification and characterization of bioactive metabolite

3.5.1 Purification of bioactive metabolite

Streptomyces species represent prolific sources of bioactive secondary metabolites with significant antimicrobial potential, and their fermentation broths contain complex mixtures of compounds that exhibit promising activity against antibiotic-resistant pathogens such as MRSA. However, the isolation and purification of specific bioactive compounds from these complex fermentation matrices remains a critical challenge for natural product discovery and development. Therefore, we systematically purify bioactive metabolites from the most potent *Streptomyces* isolate through sequential extraction and chromatographic separation techniques. Fermentation of the isolate in starch nitrate broth yielded a cell-free filtrate (CFF) with notable antibacterial activity against MRSA. Following filtration and centrifugation, ethyl acetate extraction produced a concentrated crude extract with a viscous, pale-yellow appearance. The extract exhibited strong antimicrobial activity in preliminary agar well diffusion assays, confirming that active compounds were successfully recovered from the fermentation broth. Thin-layer chromatography analysis of the crude extract revealed multiple separation bands; however, a single dominant band was consistently observed under UV illumination at 254 nm. This band exhibited a distinct brown fluorescence and an Rf value of 0.72 when developed in the diethyl ether: n-hexane (7:3, v/v) solvent system. The intensity and clarity of this band suggested a major metabolite with a relatively non-polar character. The fluorescent compound was carefully excised, eluted with methanol, and dried to obtain a purified bioactive fraction. These results demonstrate successful isolation of a major bioactive metabolite from *Streptomyces* fermentation broth, providing a foundation for further structural characterization and antimicrobial evaluation of this promising natural product candidate.

3.5.2 Characterization of purified bioactive metabolite using GC-MS and HPLC

Bioactive metabolites from microbial sources have emerged as promising candidates for therapeutic applications, and extensive research has demonstrated their potential antimicrobial and anticancer properties across diverse pathogenic targets. However, comprehensive characterization of spe-

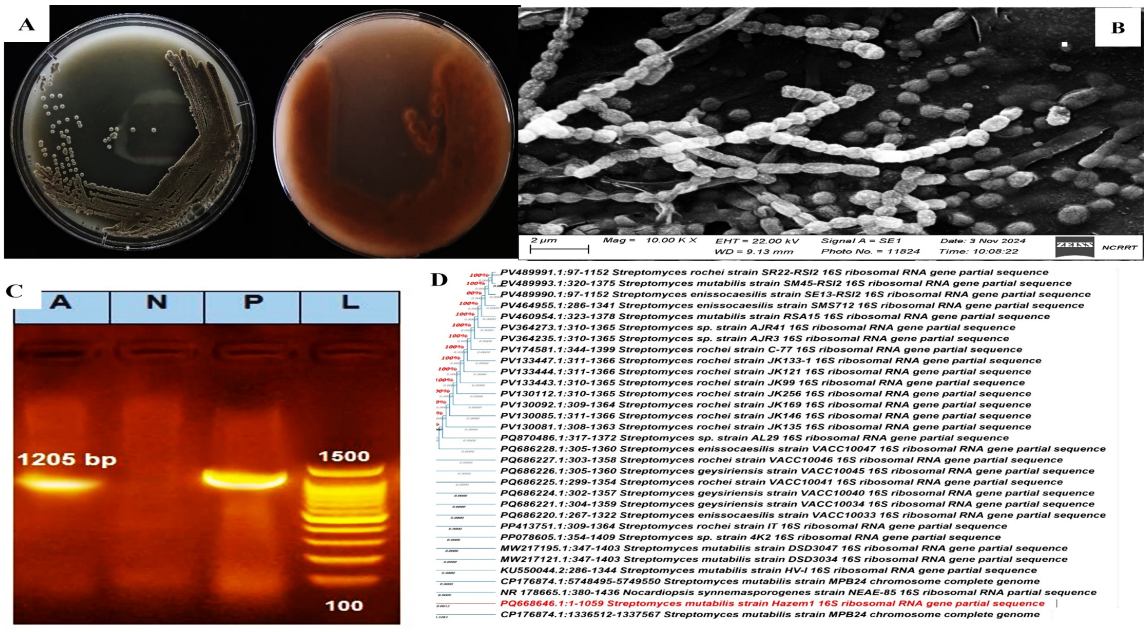


Figure 4. Identification of Actinomycetes isolate 2 (A) growth on ISP-4 medium, (B) scanning electron microscope (C) gel electrophoresis of PCR product of 16S and (D) Neighbor-joining tree based on 16S rDNA sequences showing the relationship between the isolate 2 and the related type-species of the genus *Streptomyces*.

cific compound

profiles and their targeted biological activities remains challenging, particularly in identifying predominant bioactive constituents and quantifying their therapeutic potential. Therefore, we conducted GC-MS analysis of methanol-derived bioactive metabolites to identify and characterize the major bioactive compounds and evaluate their antimicrobial and anticancer properties through comparative analysis with existing literature. As shown in figure 5 and Table 3 GC-MS analysis identified 9-Octadecenamide, (Z)- (22.25%) as the predominant compound, followed by 1-Docosene (12.1%) and 9-Hexacosene (8.51%). In silico studies have indicated that 9-Octadecenamide, (Z)- possesses significant antioxidant and antimicrobial properties (Zhang, Ding, et al., 2024). This compound has demonstrated antibacterial activity against Gram-positive bacteria, including *Staphylococcus epidermidis* and *Staphylococcus aureus*, as well as Gram-negative bacteria such as *Enterobacter aerogenes* and *Klebsiella pneumoniae*, and exhibits antifungal activity against *Candida krusei* (Sharma and Manhas, 2019) Consistent with these findings, Manikandan et al. (2019) reported that bioactive metabolites extracted with ethyl acetate from *Streptomyces* species exhibited broad-spectrum antimicrobial activity against clinically significant pathogens, including *S. aureus* MTCC 3160, *Bacillus pumilus* NCIM 2327, MRSA, Escherichia coli MTCC 1698, ESBL-producing *E. coli*, *Shigella flexneri* MTCC 1457, Proteus vulgaris, and *Enterobacter cloacae*. Furthermore, spectroscopic analysis revealed that 6-Octadecenoic acid (Z), another major metabolite, exhibits pronounced anticancer activity against MCF-7 breast cancer cells, with an IC_{50} value of 9.5 µg/mL Aftab et al. (2015) and Osama et al. (2022). These findings establish a comprehensive profile of bioactive metabolites with demonstrated therapeutic potential against multiple pathogenic targets.

HPLC–UV/Vis analysis of the purified bioactive metabolite from *Streptomyces mutabilis* revealed the presence of twelve phenolic compounds, including syringic acid (41.93%, RT 6.29 min), ellagic acid (16.49%, RT 3.55 min), ferulic acid (12.68%, RT 9.36 min), quercetin (10.49%, RT 17.48 min), coumaric acid (4.01%, RT 8.59 min), cinnamic acid (4.12%, RT 19.19 min), ellagic acid (4.61%, RT 7.52 min), naringenin (3.08%, RT 10.28 min), kaempferol (3.00%, RT 20.79 min), caffeic acid (2.60%, RT 5.79 min), vanillin (1.95%, RT 8.96 min), and chlorogenic acid (1.89%, RT 4.33 min). Among these, syringic acid was the most abundant, followed by ellagic acid, ferulic acid, and quercetin. Compound identities were confirmed by comparison with authentic reference standards, as illustrated in Fig. 6 A and B and summarized in Table 4. Comparable phenolic profiles have been reported in other *Streptomyces* species. For example, the ethyl acetate extract of *Streptomyces* coeruleofuscus exhibited notable anticancer activity against TNBC MDA-MB-468 cells, achieving inhibition rates of $62.76 \pm 0.62\%$, $62.67 \pm 0.93\%$, and $58.07 \pm 4.82\%$ at concentrations of 25, 50, and 100 µg/mL, respectively. HPLC–UV/Vis analysis of this extract identified nine phenolic compounds, including gallic acid, sinapic acid, p-coumaric acid, cinnamic acid, trans-ferulic acid, syringic acid, chlorogenic acid, ellagic acid, and epicatechin (Sommer et al., 2008). Syringic acid, the dominant compound in the present study, has been highlighted for its anticancer properties; it does not inhibit mammalian polymerases but has been shown to suppress cancer cell proliferation. For instance, syringic acid extracted from proso millet (*Panicum miliaceum* L.) partially inhibited the growth of human breast (MDA) and liver (HepG2) cancer cell lines (El-Sayed, 2012).

Similarly, HPLC–UV/Vis analyses of extracts from other *Streptomyces* strains identified phenolic compounds such as gallic acid, chlorogenic acid, vanillic acid, trans-ferulic acid, ellagic acid, and cinnamic acid, which are recognized for their antimicrobial and antioxidant activities and possess potential therapeutic applications against antibiotic-resistant pathogens and oxidative stress-related disorders (Haq et al., 2024). Additionally, vanillic acid and trans-ferulic acid demonstrated significant antibacterial and antifungal activities, further underscoring the potential of *Streptomyces* species as natural sources of clinically relevant bioactive compounds (Dos Reis et al., 2019).

3.6 Anti-MRSA of activity of purified bioactive metabolite

Methicillin-resistant *Staphylococcus aureus* represents a major clinical threat in healthcare settings, and natural products from *Streptomyces* species have historically served as important sources of antibacterial compounds. However,

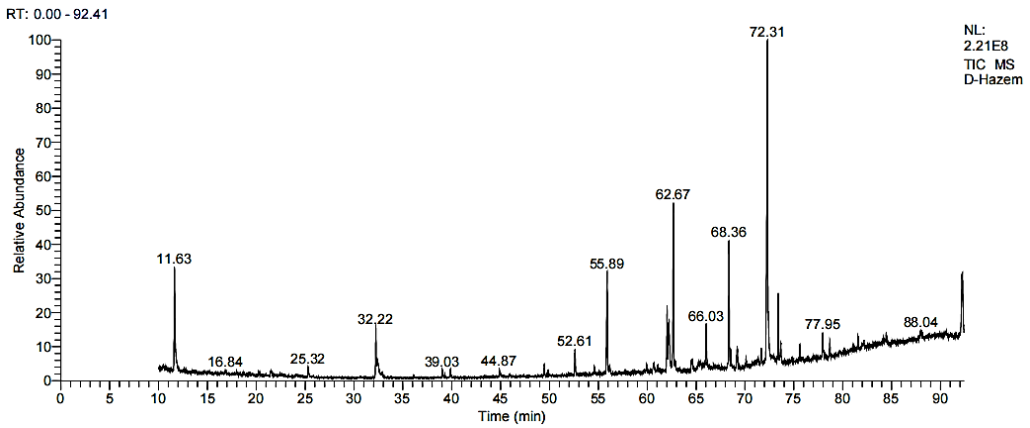


Figure 5. GC-MS chromatogram of purified bioactive metabolite.

Table 3. Chemical profiling of the purified bioactive metabolite by GC-MS.

Peak	Rotation time	Contents %	Compound	Molecular formula	Molecular weight
1	11.63	6.28	Cyclohexanol, 1-methyl-4-(1-methylel)	$C_{12}H_{20}O_2$	196
2	25.32	0.63	á-Terpinol acetate	$C_{12}H_{20}O_2$	196
3	32.23	2.77	1-Dodecanamine, N, N-dimethyl	$C_{14}H_{31}N$	213
4	32.40	0.68	1-Dodecanamine, N, N-dimethyl	$C_{14}H_{31}N$	213
5	39.03	0.56	trans-13-Octadecenoic acid	$C_{13}H_{34}O_2$	282
6	39.85	0.56	1-Tetradecanamine, N, N-dimethyl	$C_{164}H_{35}N$	241
7	44.87	0.52	Cyclononasiloxane, octadecamethyl	$C_{18}H_{54}O_9Si_9$	666
8	49.47	0.79	1-Eicosene	$C_{20}H_{40}$	280
9	52.61	1.64	Phenethylamine, N-benzyl-á-methyl	$C_{16}H_{19}N$	225
10	55.89	7.64	1-Tetracosen	$C_{24}H_{48}$	236
11	62.03	4.18	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	370
12	62.24	3.93	Phenol, 2,2′-methylenebis [6-(1 ,1-dimethylethyl)-4-m ethyl	$C_{23}H_{32}O_2$	340
13	62.66	12.01	1-Docosene	$C_{22}H_{44}$	308
14	64.51	0.53	Cyclodecasiloxane, eicosamethyl	$C_{20}H_{60}O_{10}Si_{10}$	740
15	64.59	0.60	1,3-Dipalmitin, TMS derivative	$C_{38}H_{76}O_5Si$	640
16	66.02	3.23	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390
17	68.37	8.51	9-Hexacosene	$C_{26}H_{52}$	364
18	68.57	1.04	Cyclodecasiloxane, eicosamethyl	$C_{20}H_{60}O_{10}Si_{10}$	740
19	69.20	1.66	2H-anthra [2,1-cip yran-4,11(1H,8H)] Dione	$C_{21}H_{20}O$	368
20	70.11	0.55	Fumaric acid, hexadecyl 2-hexyl ester	$C_{26}H_{48}O_4$	424
21	71.66	1.03	1,3-Benzenedicarboxylic acid	$C_{24}H_{38}O_4$	390
22	72.30	22.25	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281
23	73.40	4.12	Octacosanol	$C_{28}H_{58}O$	410
24	73.66	1.50	–Heptatriacotanol	$C_{37}H_{76}O$	536
25	75.60	1.03	Cyclodecasiloxane, eicosamethyl	$C_{20}H_{60}O_{10}Si_{10}$	740
26	77.96	1.61	17-Pentatriacontene	$C_{35}H_{70}$	490
27	78.69	1.08	Cyclodecasiloxane, eicosamethyl	$C_{20}H_{60}O_{10}Si_{10}$	740
28	82.16	1.13	Oleic acid, eicosyl ester	$C_{38}H_{74}O_2$	562
29	84.45	0.81	9,12-Octadecadienoic acid	$C_{27}H_{54}O_4Si_2$	492
30	92.25	5.69	Quercetin, 5TMS derivative	$C_{30}H_{50}O_7Si_5$	662

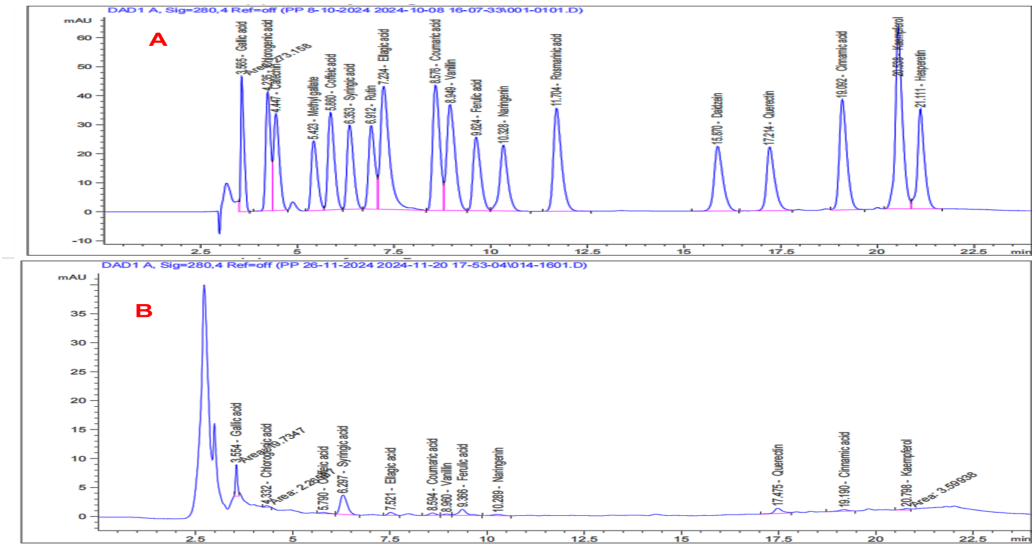


Figure 6. HPLC chromatogram (A) standards compounds used in identification and (B) compounds of purified bioactive metabolite of *Streptomyces mutabilis*.

Table 4. HPLC chromatogram of the purified bioactive metabolite of *Streptomyces mutabilis*.

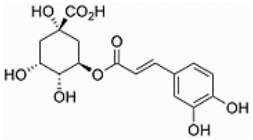
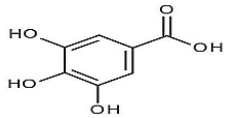
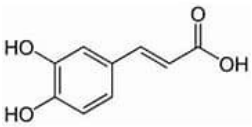
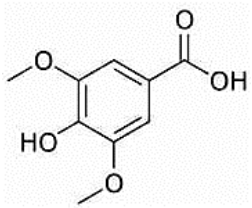
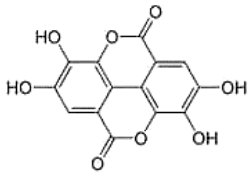
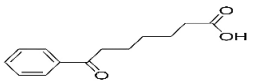
No	RT	%	Name/Molecular formula	Chemical structure	Biological activities	References
1	4.332	1.8930	Chlorogenic acid $C_{16}H_{18}O_9$		Antioxidant, anti-inflammatory, liver kidney nervous system protection, anti-bacterial, anti-tumor,	(Haq et al., 2024), (Khalid Mu et al., 2023)
2	3.554	16.487	Gallic acid $C_7H_6O_5$		Antioxidant, anti-inflammatory, anti-tumor, anti-bacterial, anti-diabetes, anti-obesity, and anti-microbial	(Haq et al., 2024), (Khalid Mu et al., 2023)
3	5.790	2.6093	Coffeic acid $C_9H_8O_4$		Antioxidant, anti-inflammatory, anti-inflammatory, anticancer, antibacterial, antiviral, Parkinson's Disease, antidepression, anti-obesity and anti-diabetic	(Haq et al., 2024), (Khalid Mu et al., 2023)
4	6.297	41.938	Syringic acid $C_9H_{10}O_5$		Antioxidant, anti-inflammatory and anticancer	(Haq et al., 2024), (Khalid Mu et al., 2023)
5	7.521	4.6104	Ellagic acid $C_{14}H_6O_8$		Antidiabetic, antibacterial, antiparasitic, antiviral, antioxidant, anti-inflammatory, anti-aging, cardio-protective, neuroprotective, and immunomodulatory properties.	(Haq et al., 2024), (Khalid Mu et al., 2023)
6	8.594	4.0104	Coumaric acid $C_9H_8O_3$		Antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, antibacterial, antiparasitic, and antiviral	(Haq et al., 2024), (Khalid Mu et al., 2023)

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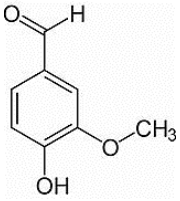
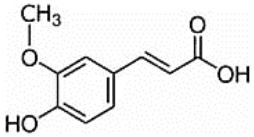
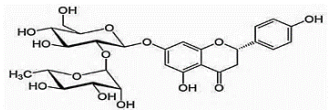
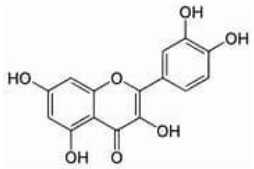
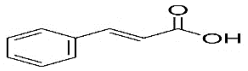
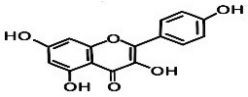
Table 5. Continued of Table 4.

No	RT	%	Name/Molecular formula	Chemical structure	Biological activities	References
7	8.960	1.9516	Vanillin $C_{87}H_8O_3$			(Haq et al., 2024), (Khalid Mu et al., 2023)
8	9.366	12.688	Ferulic acid $C_{10}H_{10}O_4$		Antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, antiviral, and antibacterial.	(Haq et al., 2024), (Khalid Mu et al., 2023)
9	10.289	3.0886	Naringenin $C_{27}H_{32}O_9$		Antioxidant, anti-inflammatory and anticancer	(Haq et al., 2024), (Khalid Mu et al., 2023)
10	17.475	10.495	Quercetin $C_{15}H_{10}O_7$		Antioxidant, anti-inflammatory, anticancer, antibacterial antiviral, antihypertensive and vasodilator effects.	(Haq et al., 2024), (Khalid Mu et al., 2023)
11	19.190	4.1205	Cinnamic acid $C_9H_8O_2$		Antioxidant, anti-inflammatory, anti-inflammatory, anticancer, antibacterial and antiviral.	(Haq et al., 2024), (Khalid Mu et al., 2023)
12	20.798	3.0071	Kaempferol $C_{15}H_{10}O$			(Haq et al., 2024), (Khalid Mu et al., 2023)

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the emergence of multidrug-resistant MRSA strains has created an urgent need for novel therapeutic agents with potent antimicrobial activity. Therefore, we investigated the antibacterial potential of a purified bioactive metabolite isolated from *Streptomyces mutabilis* against clinical MRSA isolates. The purified bioactive metabolite exhibited potent antibacterial activity against all tested clinical MRSA isolates, demonstrating a minimum inhibitory concentration of 10.0 µg/mL and a minimum bactericidal concentration of 20.0 µg/mL. These findings suggest that *Streptomyces mutabilis*-derived metabolites represent promising candidates for developing new antimicrobial therapies against resistant staphylococcal infections. These results are consistent with findings by Belghit et al. (2016) who demonstrated that ethyl acetate extracts of *S. mutabilis* displayed strong antibacterial activity against MRSA, also with an MIC of 10.0 ± 0.0 µg/mL. Similarly, Zhang et al. (2014) reported that bioactive metabolites from *Streptomyces* sp. inhibited the growth of five Gram-positive bacteria, with MIC values ranging from 0.125 to 16 µg/mL. In addition, Sharma and Manhas (2019) isolated *Streptomyces* antibioticus strain M7 from soil, which produced antimicrobial compounds effective against vancomycin-resistant enterococci (VRE) and MRSA, with MICs of $1.95 - 2.25$ µg/mL and $3.5 - 4.0$ µg/mL, respectively. Collectively, these studies underscore the remarkable capacity of *Streptomyces* species to synthesize a diverse array of natural anti-MRSA compounds. Such bioactive strains have been recovered from a variety of ecological niches-including terrestrial soils, tropical forests, marine sediments, and symbiotic associations with other organisms-highlighting the genus as a prolific and versatile source for the discovery of novel antimicrobial agents (Kemung et al., 2018).

3.7 Antibiofilm activity

MRSA biofilms represent a major virulence factor in antibiotic-resistant infections, and these structures confer protection against both antimicrobial agents and host immune defenses, making infections difficult to eradicate (Elbestawy et al., 2023). However, conventional antibiotics demonstrate limited efficacy against biofilm-associated MRSA infections due to their inherent resistance mechanisms and reduced penetration through biofilm matrices. Therefore, we systematically evaluated the antibiofilm activity of bioactive metabolites derived from *S. mutabilis* against MRSA strains to identify novel therapeutic approaches for biofilm disruption. Our results demonstrated that these metabolites effectively inhibited biofilm formation at sub-MIC concentrations of 1/8, 1/4, and 1/2 MIC. The extent of inhibition was concentration-dependent, with 1/2 MIC exhibiting the most pronounced suppression of biofilm formation, followed by 1/4 and 1/8 MIC, and all effects were statistically significant ($p < 0.0001$) as shown in figure 7. Our study contributes to the growing body of evidence supporting *Streptomyces*-derived metabolites as promising candidates for developing selective and safe anti-biofilm agents capable of disrupting biofilm development and compromising structural integrity in MRSA infections, potentially overcoming the therapeutic challenges posed by biofilm-associated antibiotic resistance. These findings align with previous research demonstrating that secondary metabolites produced by *Streptomyces* species, such as streptorubin B, possess potent antibiofilm properties (Hughes et al., 2010). Similarly, ethyl acetate extracts from *Streptomyces* sp. SBT343 significantly inhibited biofilm formation in multiple *Staphylococcus* species, including MRSA strain USA300, with a maximum inhibitory concentration of 125 µg/mL Remarkably, these compounds exhibited selective antibiofilm activity against *Staphylococcus* species without affecting the growth of Gram-negative bacteria such as Pseudomonas aeruginosa and displayed negligible cytotoxicity at therapeutically relevant concentrations (Kemung et al., 2018; Balasubramanian et al., 2017). The antibacterial and antibiofilm activities of *Streptomyces mutabilis* metabolites may involve multiple mechanisms, including disruption of bacterial cell walls and membranes, interference with quorum sensing pathways, inhibition of biofilm matrix formation, and modulation of virulence-related gene expression. Considering these potential mechanisms provides a more mechanistic understanding of our findings and strengthens the scientific rationale underlying the observed bioactivities (Kalaba et al., 2024b).

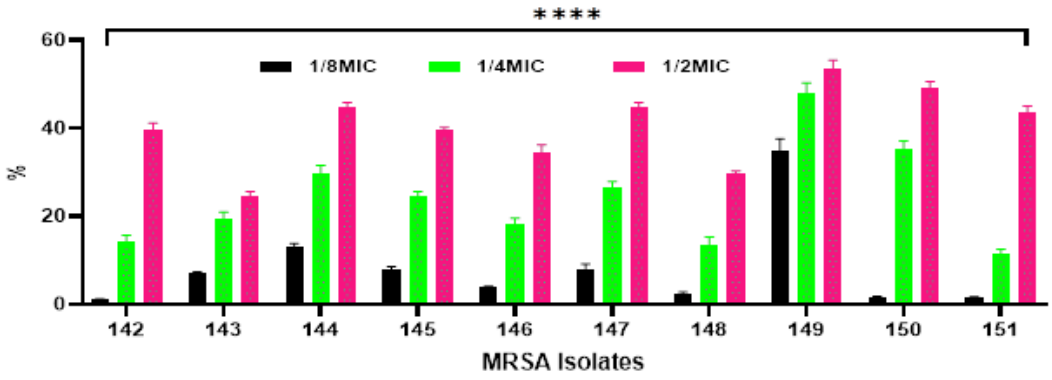

Figure 7. Antibiofilm activity of bioactive metabolite against MRSA strains (**** $p < 0.0001$).

3.8 Cytotoxicity and anticancer activity of the purified bioactive metabolite

The search for natural anticancer agents remains a key priority in drug discovery, and *Streptomyces* species are widely recognized as prolific sources of bioactive metabolites with demonstrated antitumor activity (El-Sayed, 2012). Therefore, we evaluated the cytotoxic activity of a purified bioactive metabolite against normal (HFB-4) and cancerous (HepG2, MCF-7, and PC-3) cell lines to determine its therapeutic potential and selectivity profile. Treatment with the purified bioactive metabolite at varying concentrations for 24 hours induced distinct morphological alterations at the cell surface and within the cytoskeleton, with structural changes consistent with reduced cell viability compared to untreated controls. MTT assay results demonstrated significant decline in cell viability beginning at concentrations of 100 µg/mL and above. The half-maximal inhibitory concentration (IC_{50}) values were calculated as 280.0 ± 2.29 µg/mL for HFB-4, 121.31 ± 1.28 µg/mL for HepG2, 73.4 ± 0.19 µg/mL for PC-3, and 116.6 ± 1.08 µg/mL for MCF-7, with the highest cytotoxic potency observed against PC-3 cells. Morphological hallmarks of cytotoxicity included reduced cell volume due to loss of intracellular ions and proteins resulting from altered sodium and potassium permeability. Necrotic cells exhibited nuclear swelling, chromatin flocculation, and loss of nuclear basophilia, whereas apoptotic cells displayed cell shrinkage, nuclear condensation, and nuclear fragmentation as shown in figure 8. These findings demonstrate selective cytotoxicity against cancer cells with preferential activity against PC-3 prostate cancer cells, supporting the potential therapeutic value of this *Streptomyces*-derived metabolite. Several potent anticancer compounds have been isolated from *Streptomyces* species, including 1-(1H-indol-3-yl)-propane-1,2,3-triol and chromomycin SA from *Streptomyces* sp. KML-2, which demonstrated strong cytotoxicity against MCF-7 and HeLa cell lines with IC_{50} values ranging from 0.97 to 12.6 µg/mL (Rammali et al., 2024) and benzoxazole from *Streptomyces* sp. Tü 6176, showing pronounced activity against multiple cancer cell lines with IC_{50} values as low as 0.06 µg/mL (Mangzira Kemung et al., 2020). However, the cytotoxic effects and selectivity profile of many purified bioactive metabolites from *Streptomyces* remain inadequately characterized across diverse cancer cell types (Rammali et al., 2024). *Streptomyces* species have demonstrated significant potential as sources of bioactive compounds with antibacterial and anticancer properties, and previous studies have reported these activities individually across various strains. However, comprehensive integrated evaluation combining antibacterial, antibiofilm, and cytotoxic activities with detailed chemical characterization of metabolites from newly isolated soil-derived strains remains limited, particularly for multifunctional approaches targeting MRSA infections and biofilm-associated pathogenicity. Therefore, we conducted an integrated evaluation of a newly isolated *Streptomyces mutabilis* strain, uniquely linking isolation, bioactivity assessment, and identification of key bioactive compounds, particularly syringic acid. This comprehensive approach highlights the potential of this strain as a multifunctional source of natural compounds for combating MRSA infections and biofilm-associated pathogenicity, representing a novel contribution not reported in prior studies.

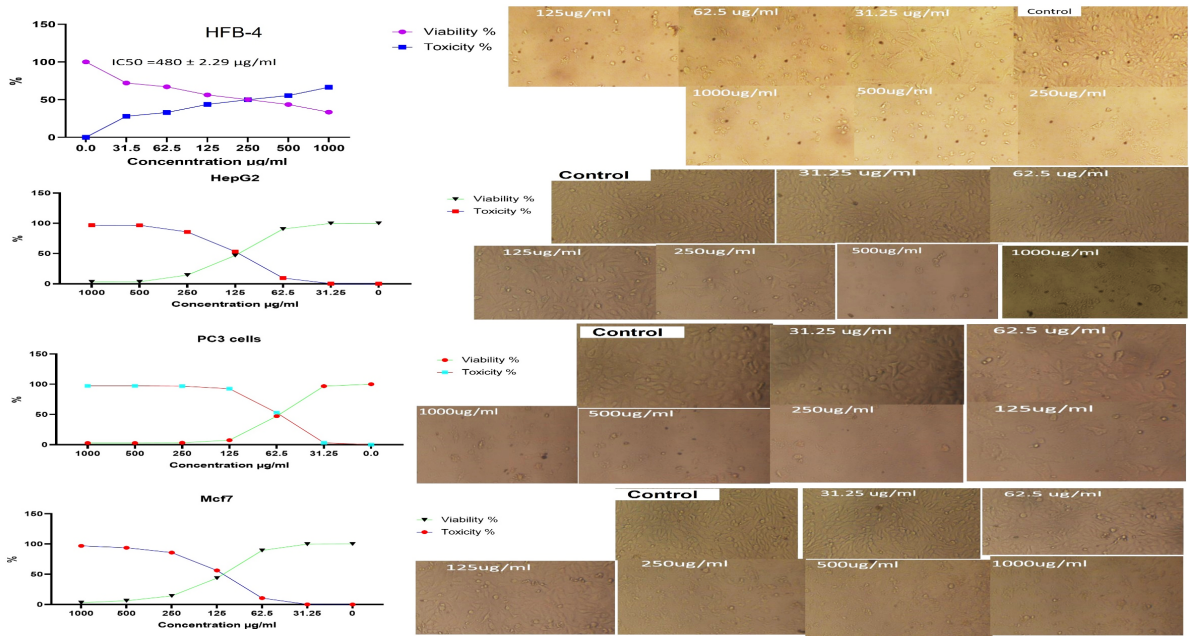

Figure 8. Cytotoxicity and anticancer activity of the bioactive metabolite against normal (HFB-4) and cancerous (HepG2, MCF-7, and PC-3) cell lines treated with different concentrations under an inverted microscope using MTT assay (**** $p < 0.0001$).

4. Conclusion

Staphylococcus aureus, particularly methicillin-resistant S. aureus, continues to pose a significant clinical challenge owing to its capacity for biofilm formation and multidrug resistance. The present study highlights the discovery of a novel actinobacterial isolate; soil-derived *S. mutabilis* was identified as a promising source of bioactive metabolites, with syringic acid recognized as a major constituent exhibiting potent antibacterial and antibiofilm activities, along with moderate anticancer effects. These results demonstrate the medicinal value of *S. mutabilis* metabolites and establish a foundation for future mechanistic and in vivo investigations aimed at optimizing their application against drug-resistant infections. Moreover, this work highlights the invaluable role of soil microbial diversity in the discovery of novel natural bioactive compounds to address the escalating threat of antimicrobial resistance. Future research should assess their efficacy against a wider range of pathogens and include comprehensive in vivo studies to evaluate safety, biocompatibility, pharmacokinetics, and therapeutic performance.

Ethical Approval

The study was conducted on human subjects and/or human data, adhering to the principles outlined in the Helsinki Declaration, and referenced the Institutional Review Board (IRB) approval. The Institutional Review Board of National Cancer Institute (NCI), Cairo University, Egypt, authorized all protocols and procedures (IRB number: IRB-00004025; approval number: CB2410-102-083-192). Each participant signed their written informed consent form before enrolling in this study.

Authors contributions

H.A.S. conceptualization, examination, and original draft writing G.M.S. and S.A.M. conceptualization, conceived the presented idea, co-wrote the paper, and supervised the research; Z.A.F. conceptualization, examination, and original draft writing; M.H.S. conceptualization, investigation, original draft writing, supervision. All authors have read and approved the version of the manuscript that has been published.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request. This nucleotide sequence of the 16S rRNA gene has been deposited in the NCBI gene bank, https://www.ncbi.nlm.nih.gov/search/all/?term=PQ668646) assigned the accession numbe (PQ668646).

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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