

Bioactivity and Secondary Metabolites Profiling of *Kitasatospora* sp. TC-ARCL7 Isolated from *Curcuma longa* L. Rhizosphere

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Abstract

Actinomycetes are important sources of bioactive compounds with pharmaceutical potential. *Kitasatospora* sp. TC-ARCL7, isolated from the rhizosphere of *Curcuma longa* L., has previously shown anticancer activity against the T47D cell line, but its broader biological activities remain unexplored. This study evaluated the anticancer, antimicrobial, and antioxidant activities of its ethyl acetate extract. Cytotoxic activity was assessed against T47D, HepG2, HeLa, and Vero cell lines, while antimicrobial activity was determined against selected bacterial and fungal pathogens using MIC analysis. Antioxidant activity was evaluated using the DPPH and FRAP assay. The extract exhibited potent cytotoxic activity, with IC₅₀ values of 0.20 ± 0.04 (SI: 3883.03 ± 476.79), 2.64 ± 0.46 (SI: 301.28 ± 67.38), and 2.33 ± 0.39 (SI: 328.86 ± 23.37) $\mu\text{g/mL}$ against T47D, HepG2, and HeLa cells, respectively. The *Kitasatospora* sp. TC-ARCL7 isolate inhibited the growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. In contrast, the ethyl acetate extract showed no inhibitory zone against *Pseudomonas aeruginosa* and *C. albicans*. The extract exhibited antioxidant activity predominantly through electron-transfer mechanisms, as indicated by its strong FRAP response (118.70 ± 18.8 mM Fe(II)/g) despite weak DPPH scavenging (IC₅₀ = 513.35 $\mu\text{g/mL}$). GC-MS analysis identified several compounds previously reported to possess anticancer, antimicrobial, and antioxidant activities. The high selectivity index and potent cytotoxic activity against T47D cells suggest that the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 is a promising source of bioactive compounds for further research into anticancer drug discovery.

Keywords

Kitasatospora, Rhizosphere, *Curcuma longa*, Anticancer, Antimicrobial, Antioxidant

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1. INTRODUCTION

Over the years, scientists have focused on identifying natural sources for treating human diseases (Bhosale et al., 2016). Compounds with antimicrobial, anticancer, antioxidant, anti-inflammatory, and other biological activities have been extensively explored (El-Fayoumy et al., 2021). Antimicrobial resistance (AMR) has become a major global concern due to the inappropriate use of antibiotics and the emergence of resistance mechanisms in pathogenic microorganisms, which reduce treatment efficacy and increase disease persistence (Ningsih and Sulistyan, 2018; Rajivgandhi et al., 2018). Another important research focus is the discovery of anticancer compounds that selectively target cancer cells while minimizing toxicity

to normal cells, thereby improving their therapeutic potential (Calderón-Montañó et al., 2021; Krzywik et al., 2020).

Although chemically synthesized compounds have been widely used, exploring naturally derived bioactive compounds remains important because they generally cause fewer side effects (Mishra et al., 2020; Silfarohana et al., 2025). Actinomycetes are filamentous Gram-positive bacteria with remarkable metabolic diversity and are widely distributed in terrestrial and aquatic environments. They are well known for producing bioactive secondary metabolites with antimicrobial, antifungal, and anticancer activities (Gurung and Rai, 2021). Rhizospheric and endophytic actinomycetes have attracted considerable attention because they may produce bioactive metabolites similar

to those of their host plants (Damam et al., 2016). Previous studies have shown that endophytic microorganisms can synthesize metabolites analogous to those produced by their host plants (Shabira et al., 2022).

Curcuma longa is one of the most widely used medicinal plants among several ethnic groups in Indonesia (Wahyono et al., 2017) and exhibits antimicrobial and anticancer properties (Adahoun et al., 2017; Tomeh et al., 2019). *Kitasatospora* sp. TC-ARCL7 was successfully isolated from the rhizosphere of *Curcuma longa* collected in Jumantono, Karanganyar, Central Java, Indonesia (Sari et al., 2025), and has been registered in the NCBI database under accession number PP212015. Our previous study demonstrated that *Kitasatospora* sp. TC-ARCL7 exhibits cytotoxic activity against the T47D breast cancer cell line (Sari et al., 2025). However, information regarding its broader biological activities and metabolite composition remains unavailable.

Therefore, the present study aimed to comprehensively evaluate the cytotoxic, antimicrobial, and antioxidant activities of the ethyl acetate extract together with metabolite profiling using TLC and GC-MS, to provide a more comprehensive assessment of its pharmaceutical potential. Anticancer activity was assessed against T47D, HepG2, and HeLa cells, with Vero cells used as a normal cell line. Antimicrobial activity was evaluated against selected bacterial and fungal pathogens, followed by MIC determination, whereas antioxidant activity was assessed using the DPPH and FRAP assays. In addition, the secondary metabolite profile of the extract was characterized using TLC and GC-MS.

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used in this study included the *Kitasatospora* sp. TC-ARCL7 isolate and its crude ethyl acetate extract. The media used for microbial growth consisted of Starch Casein Agar (SCA) containing soluble starch (10 g/L), casein (0.3 g/L), KNO_3 (2 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 g/L), K_2HPO_4 (2 g/L), NaCl (2 g/L), CaCO_3 (0.02 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g/L), and agar (18 g/L), as well as Starch Casein Broth (SCB), ISP2 medium, Nystatin antifungal agent, Mueller-Hinton Agar (MHA), and Nutrient Broth. Analytical-grade ethyl acetate was used for extraction. Materials used for the antimicrobial assay included bacterial test organisms (*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*), DMSO, blank discs (OxoidTM), chloramphenicol discs, and nystatin. The antioxidant assay employed DPPH and FRAP reagents as the test reagents. Materials used for TLC analysis included silica gel aluminum sheets, chloroform, and methanol. The instruments used in this study included a biosafety cabinet (BSC), autoclave, shaker incubator, incubator, porcelain dish, Erlenmeyer flask, analytical balance, hot plate magnetic stirrer, Thermo Fisher Multiskan SkyHigh Microplate Spectrophotometer, and GC-MS system (Thermo Scientific GC Trace 1310 coupled with an ISQ 7000 MS Detector).

2.2 Methods

2.2.1 Actinomycete Isolate

Kitasatospora sp. TC-ARCL7, an actinomycete isolated from the rhizosphere of *Curcuma longa* L. in Tawangmangu, Karanganyar, Central Java, Indonesia, was used in this study. The isolate was maintained in Starch Casein Agar (SCA) and in ISP2 medium for actinomycetes (Kumar et al., 2013).

2.2.2 Primary Screening of Antimicrobial Activity

To assess the antimicrobial properties of *Kitasatospora* sp. TC-ARCL7, the isolate was streaked across half of a Mueller-Hinton Agar (MHA) plate and incubated for 8 days at 30°C to promote actinomycete growth and metabolite release into the medium. The bacterial test organisms (*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*) were streaked perpendicularly and incubated at 37°C. The plate was examined for inhibition of bacterial growth after 24 hours.

2.2.3 Production and Extraction of Secondary Metabolites

Secondary metabolites were extracted using our method (Sari et al., 2025). Actinomycete starter cultures were prepared by inoculating 20 mL of Starch Casein Broth (SCB) with 1% (v/v) glycerol stock of *Kitasatospora* sp. TC-ARCL7 and incubating at 28–30°C and 170 rpm for 72 h. Then, 1% (v/v) starter inoculum was transferred into 200 mL of SCB in a 500-mL Erlenmeyer flask and incubated at 28–30°C and 170 rpm for 10 days. Following incubation, the supernatant containing metabolites and cell biomass was collected by centrifugation at 6000 rpm for 15 minutes. The supernatant was extracted with an equal volume of ethyl acetate (1:1) by shaking on an orbital shaker overnight to extract the metabolites, then allowed to stand until phase separation occurred. The ethyl acetate phase was collected and evaporated at 40°C to obtain the crude extract. From 200 mL of fermentation broth, 8.25 mg of crude ethyl acetate extract was obtained, corresponding to 41.25 mg/L culture (4.1% yield). The crude extract was stored at -20°C before use. The crude extract used for all bioactivity assays was obtained by pooling extracts from several independent fermentation batches to ensure sufficient material for analysis. Therefore, the bioactivity assays were performed using a single pooled extract.

2.2.4 Secondary Screening of Antimicrobial Activity (Disc Diffusion)

Disc diffusion screening was performed against the test microorganisms *B. subtilis*, *E. coli*, *S. aureus*, *P. aeruginosa*, and *C. albicans*. Each test microorganism was grown overnight in Nutrient Broth and adjusted to a 0.5 McFarland Standard. The microorganisms were plated as a lawn on Mueller-Hinton Agar using a sterile cotton swab. Blank discs (OxoidTM, 6 mm) were loaded with 15 µL of ethyl acetate extract from *Kitasatospora* sp. TC-ARCL7 at 20, 200, and 2000 µg/mL, diluted in DMSO. The discs were then placed on the agar and incubated for 24 hours at 37°C. Positive controls were incorporated into the

tests using chloramphenicol discs for bacteria and nystatin for *Candida albicans*. Antimicrobial activity was determined by measuring the zone of inhibition. Each assay was conducted in triplicate as technical replicates, and the results are presented as mean $\pm$ SD.

2.2.5 MIC and MBC Determination

The Minimum Inhibitory Concentration (MIC) of the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 followed previous research with slight modifications (Gebreyohannes et al., 2013; Swebosci et al., 2023). Test microorganisms *B. subtilis*, *E. coli*, *S. aureus*, and *P. aeruginosa* were grown overnight at 37°C and adjusted to an OD600 of 0.1 as the test inoculum in Nutrient Broth. Two-fold serial dilutions of the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 were prepared at 5000 $\mu\text{g/mL}$ and dispensed into rows 1–12 of a 96-well microplate at 50 μL in triplicate. 50 μL of test inoculum was added to each well. For growth and sterility controls, 100 μL of test inoculum and sterile nutrient broth were used, respectively. After 24 hours of incubation at 37°C, turbidity was measured using a Thermo Fisher Multiskan SkyHigh Microplate Spectrophotometer at 600 nm. The concentration at which no increase in turbidity was observed was determined as the MIC of the ethyl acetate extract. Each assay was conducted in triplicate as technical replicates.

The Minimum Bactericidal Concentration (MBC) of the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 was determined by inoculating 0.1 mL of the broth from the 96-well microplate onto Mueller-Hinton Agar at MIC levels and above. The concentration at which no colonies were observed after 24 hours of incubation was recorded as the MBC.

2.2.6 Cytotoxicity Assay

The anticancer activity of the ethyl acetate extract was evaluated against T47D (human breast cancer), HepG2 (human liver cancer), HeLa (cervical carcinoma), and Vero cell lines using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were grown in DMEM supplemented with 1% penicillin-streptomycin and 10% Fetal Bovine Serum at 37°C in 5% CO₂. Approximately 8×10^3 cells/mL were seeded into a 96-well microtiter plate and incubated for 24 hours. Each cell line was then treated with fresh media containing the ethyl acetate extract at concentrations ranging from 80 $\mu\text{g/mL}$ to 0.04 $\mu\text{g/mL}$, diluted two-fold. For the normal cell line Vero, treatment was performed at concentrations ranging from 800 $\mu\text{g/mL}$ to 0.4 $\mu\text{g/mL}$. The same doxorubicin concentrations were used as a control for each cell line. After 24 hours, the treated media were replaced with 1% MTT in DMEM, and plates were incubated for 3 hours. After formazan crystals formed, the reaction was stopped by adding 10% SDS to 0.01N NaOH. The developed color was measured with an ELISA reader at 595 nm to determine the IC₅₀ of each cell line (Ravikumar et al., 2012). Each assay was conducted in triplicate as technical replicates, and the results are presented as mean $\pm$ SD. Statistical analyses were performed using Welch's

t-test.

2.2.7 DPPH Radical Scavenging Assay

The antioxidant activity of the ethyl acetate extract was assessed using the DPPH method described by Widodo with minor modifications (Widodo et al., 2020). The DPPH solution was prepared by dissolving DPPH (2,2-diphenyl-1-picrylhydrazyl) in 100% methanol to obtain a concentration of 0.1 mM (39.4 $\mu\text{g/mL}$). The sample and control solutions were prepared in 1 mL microtubes, with six concentration series: 300, 240, 180, 120, 60, and 0 $\mu\text{g/mL}$. The sample solution consisted of the extract, methanol, and DPPH, while the control solution consisted of the extract and methanol without DPPH. The microtubes were incubated in a dark place or in an incubator at 37°C for 30 minutes. The sample and control solutions for each concentration series were placed in a 96-well microplate and read at 515 nm using a spectrophotometer. Each assay was conducted in triplicate as technical replicates, and the results are presented as mean $\pm$ SD. Radical-scavenging activity was expressed as percent inhibition and used to calculate the IC₅₀ by the formula below (A₀: the absorbance of the control solution; A₁: the absorbance of the sample solution or extract).

$$IC_{50} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

Antioxidant activity was categorized using the Antioxidant Activity Index (AAI) formula.

$$AAI = \frac{\text{final concentration of DPPH } (\mu\text{g/mL})}{IC_{50} (\mu\text{g/mL})} \quad (2)$$

The Antioxidant Activity Index value consists of low (AAI < 0.5), moderate (0.5 < AAI < 1.0), strong (1.0 < AAI < 2.0), and strongest (AAI > 2) (Scherer and Godoy, 2009).

2.2.8 FRAP Assay

The reducing capacity of the plant extracts was determined using the FRAP assay according to Widodo et al. (2019). The FRAP reagent was prepared fresh daily by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM FeCl₃ · 6H₂O solution at a volume ratio of 10:1:1. FeSO₄ · 7H₂O was used to construct the standard curve at final concentrations of 100–1,000 $\mu\text{M/mL}$. The reaction mixture in a 2 mL microtube consisted of 1,350 μL FRAP reagent and 150 μL of methanolic extract sample. The mixture was then incubated for 30 minutes at 37°C, and the absorbance was measured at 595 nm. The FRAP value was calculated as mM Fe(II)/g of sample using the ferric chloride standard curve.

2.2.9 Secondary Metabolite Profiling

The secondary metabolite profile of the crude ethyl acetate extract was determined by Thin Layer Chromatography (TLC) on a silica gel aluminum sheet, following a previously reported

method (Saraswathi et al., 2020). Rf values were calculated as follows:

$$\text{Rf value} = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}} \quad (3)$$

2.2.10 GC-MS

Metabolite profiling of the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 was performed by GC-MS (Thermo Scientific GC Trace 1310 with MS Detector ISQ 7000). The extract was diluted in ethyl acetate to 100 ppm and sonicated at room temperature for 30 minutes. The extract was then filtered through a PTFE 0.22 µm syringe filter. A 1 µL aliquot of the extract was injected into the TG-5MS GC column (Thermo Scientific) using a liquid injection syringe. The GC Front Inlet operated in splitless mode and was maintained at 220°C. Helium served as the mobile phase at a flow rate of 1.0 mL/min. The oven temperature program started at 70°C, ramped to 100°C at 20°C/min for 2 min, then to 240°C at 5°C/min for 2 min, and finally to 300°C at 5°C/min for 5 min. The MS transfer line and ion source temperatures were 250°C and 300°C, respectively, and mass spectral detection was performed over a mass range of 40 to 600 amu. Raw data files were processed with Chromeleon software (version 7.3), and spectra were identified using the National Institute of Standards and Technology (NIST, 2021) library.

3. RESULTS AND DISCUSSIONS

3.1 Antimicrobial Susceptibility Test

Antimicrobial activity was observed in a primary test against Gram-positive bacteria (*B. subtilis* and *S. aureus*), Gram-negative bacteria (*E. coli* and *P. aeruginosa*), and yeast (*C. albicans*), as shown in Figure ???. Compared with the growth of test microorganisms in MHA as a control, *S. aureus*, *E. coli*, *B. subtilis*, and *C. albicans* were significantly inhibited by *Kitasatospora* sp. TC-ARCL7, whereas no inhibition was detected for *P. aeruginosa*. Secondary screening using an ethyl acetate extract of the isolate revealed inhibition zones at various concentrations (Table 1). MIC and MBC were then examined only for bacteria (Table 2), as no inhibition zone was detected against *C. albicans*. The inhibition zones increased with extract concentration, indicating dose-dependent antibacterial activity. However, no inhibition was observed against *Pseudomonas aeruginosa* or *Candida albicans* after ethyl acetate extraction. Consistent with the inhibition assay, MIC and MBC analyses showed the greatest antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (MIC < 1.22 × 10⁻³ mg/mL), followed by *Staphylococcus aureus* (MIC = 4.88 × 10⁻³ mg/mL). These MIC values fall within the range considered indicative of promising antibacterial activity for crude extracts (Gonzalez-Pastor et al., 2023). In contrast, *P. aeruginosa* exhibited higher MIC (1.25 mg/mL) and MBC (5.00 mg/mL; 4× MIC) values, consistent with its intrinsic resistance, including low outer membrane permeability, multidrug efflux pumps, and diverse resistance determinants (Bubonja-šonje et al., 2020; Elfadadny et al., 2024; Karungamy et al., 2023).

Overall, the extract was more active against Gram-positive than Gram-negative bacteria, although *E. coli* remained highly susceptible. The lower susceptibility of Gram-negative bacteria is generally attributed to their lipopolysaccharide-rich outer membrane, selective porins, and active efflux pumps, which restrict antimicrobial penetration (Henderson et al., 2016; Saxena et al., 2023). The contrasting susceptibility of *E. coli* and *P. aeruginosa* further suggests that antibacterial activity depends not only on Gram classification but also on species-specific intrinsic resistance mechanisms.

Although *C. albicans* was inhibited during primary screening, no antifungal activity was detected after ethyl acetate extraction. This loss of activity may reflect poor recovery of antifungal metabolites due to their polarity, instability, or low abundance. Previous studies have shown that methanol or methanol/ethyl acetate extraction improves the recovery of antifungal metabolites (Dewi et al., 2017; Palla et al., 2018). Alternatively, synergistic interactions among metabolites in the fermentation broth may have been disrupted during solvent extraction.

3.2 Antioxidant Activity

The total electron-donating capability of the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 was quantified using the FRAP assay. The extract exhibited a high ferric-reducing capacity of 118.70 ± 1.88 mM Fe(II)/g dry extract (Table 3). Interestingly, while the extract exhibited weak DPPH radical-scavenging activity (IC₅₀ = 513.35 µg/mL, AAI = 0.07) (Table 3), it showed a strong ferric-reducing potential in the FRAP assay. This notable contrast highlights the fundamental differences in the chemical mechanisms underlying these two assays. The DPPH assay relies on both Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) to neutralize a bulky, nitrogen-centered organic radical. Its efficiency can be significantly hindered by steric hindrance or limited access to active sites within a complex microbial crude extract (Gulcin and Alwasel, 2023). In contrast, the FRAP assay strictly measures pure Single Electron Transfer (SET) kinetics, evaluating the ability of secondary metabolites to reduce the ferric-tripyridyltriazine (Fe³⁺-TPTZ) complex to its intensely blue ferrous (Fe²⁺-TPTZ) form under acidic conditions (pH 3.6) (Khemiri et al., 2009).

The high FRAP activity reveals that the secondary metabolites in the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 act predominantly as potent single-electron donors rather than direct hydrogen-donating radical scavengers. In biological systems, this electron-donating mechanism plays a crucial role in cellular defense. Transition metals such as Fe³⁺ act as major pro-oxidants that catalyze the generation of highly destructive hydroxyl radicals from hydrogen peroxide (H₂O₂) via Fenton-type reactions (Phaniendra et al., 2015). By efficiently donating electrons to reduce and stabilize pro-oxidant metal ions, the extract's bioactive compounds can halt metal-induced intracellular oxidative cascades (Moon and Shibamoto, 2009). Thus, the integration of a strong FRAP response with weak DPPH

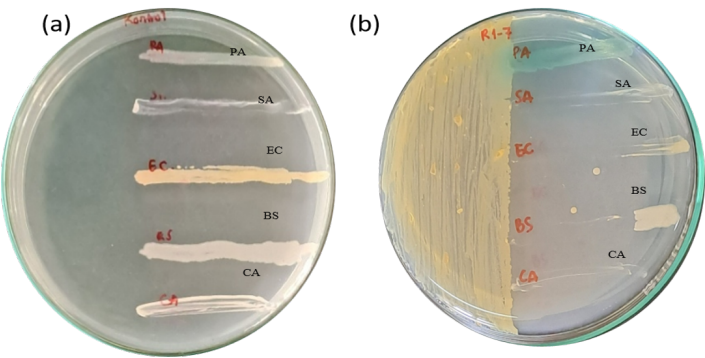


Figure 1. Primary Screening of *Kitasatospora* sp. TC-ARCL7 Against PA = *P. aeruginosa*, SA = *S. aureus*, EC = *E. coli*, BS = *B. subtilis*, and CA = *C. albicans*. a) Growth of Test Microorganisms on MHA, b) Test Microorganisms Against *Kitasatospora* sp. TC-ARCL7

Table 1. Inhibition Zone of Test Microorganisms Against Several Concentrations of Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7

Test Microorganisms	Inhibitory Zone (mm)				
	K(+) Chloramphenicol/ nystatin	K(-) DMSO	20 ug/mL	200 ug/mL	2000 ug/mL
<i>B. subtilis</i>	27.83 ± 1.47 ^a	nd	10.50 ± 0.55	16.17 ± 0.41	24.67 ± 0.52
<i>E. coli</i>	26.67 ± 1.03 ^a	nd	9.83 ± 0.41	15.83 ± 0.75	23.50 ± 0.55
<i>S. aureus</i>	28.17 ± 1.83 ^a	nd	6.83 ± 0.41	13.17 ± 0.41	24.67 ± 0.52
<i>P. aeruginosa</i>	23.33 ± 1.03 ^a	nd	nd	nd	nd
<i>C. albicans</i>	9.00 ± 0.00 ^b	nd	nd	nd	nd

Values are presented as mean ± SD from independent experiments in triplicate replication. a=Chloramphenicol used as antibacterial, b = Nystatin used as antifungal, n.d (not detected) = No inhibition zones detected

Table 2. MIC and MBC of the Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7

Test Microorganisms	MIC (mg/mL)	MBC (mg/mL)
<i>B. subtilis</i>	< 1.22 × 10 ⁻³	1.95 × 10 ⁻²
<i>E. coli</i>	< 1.22 × 10 ⁻³	2.60 × 10 ⁻²
<i>S. aureus</i>	4.88 × 10 ⁻³	1.56 × 10 ⁻²
<i>P. aeruginosa</i>	1.25	5.00

Values are presented as the mean from independent experiments in triplicate replication

scavenging indicates that *Kitasatospora* sp. TC-ARCL7 produces redox-active metabolites whose antioxidant protection is mediated specifically through electron-transfer pathways rather than organic free radical quenching.

3.3 Anticancer Activity

Anticancer activity was assessed to determine IC₅₀ values for several cell lines, as shown in Table 4. The lowest IC₅₀ was observed in the T47D cell line, 0.20 ± 0.04 µg/mL. The IC₅₀ of the extract against HepG2 and HeLa cells was approximately 10 times higher, at 2.64 ± 0.46 and 2.33 ± 0.39 µg/mL, re-

spectively. However, a very high selectivity index was achieved against T47D, HepG2, and HeLa cell lines, with an IC₅₀ of 775.01 ± 58.13 µg/mL in Vero cells. Doxorubicin, a commonly used chemotherapy drug, was used as a control and measured at 1.91 ± 0.69, 2.17 ± 0.23, and 2.63 ± 0.21 µg/mL in T47D, HepG2, and HeLa cell lines, respectively.

The extract shows promising cytotoxic activity, primarily against T47D breast cancer cells, with a much higher selectivity index than doxorubicin, a standard drug treatment (Table 4 and Figure 2). The selectivity index (SI) is the ratio of IC₅₀ values for healthy and cancer cells (Priyanto et al., 2022). These

Table 3. Radical Scavenging Activity (IC₅₀) of the Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7

Sample	DPPH Assay			FRAP Assay	
	R ²	IC ₅₀ value (μg/mL)	AAI	R ²	Value (mM Fe(II)/g)
Ethyl Acetate Extract of <i>Kitasatospora</i> sp. TC-ARCL7	0.98	513.35 ± 13.75	0.07 (weak)	0.99	118.70 ± 18.8

Values are presented as mean ± SD from independent experiments in triplicate.

Table 4. Inhibitory Concentration (IC₅₀) and Selectivity Index (SI) of the Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7 Against Several Cell Lines

Cell Lines	EA of <i>Kitasatospora</i> sp. TC-ARCL7		Doxorubicin		p-Value
	IC ₅₀ (μg/mL)	SI	IC ₅₀ (μg/mL)	SI	
Vero	775.01 ± 58.13	-	116.6 ± 5.42	-	-
T47D	0.20 ± 0.04	3883.03 ± 476.79	1.91 ± 0.69	77.74 ± 14.67	0.005**
HepG2	2.64 ± 0.46	301.28 ± 67.38	2.17 ± 0.23	50.56 ± 3.04	0.023*
HeLa	2.33 ± 0.39	328.86 ± 23.37	2.63 ± 0.21	44.51 ± 5.68	0.038*

Values are presented as mean ± SD from independent experiments. Statistical comparison between Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7 and doxorubicin was performed using Welch's t-test. **p* < 0.05, ***p* < 0.01

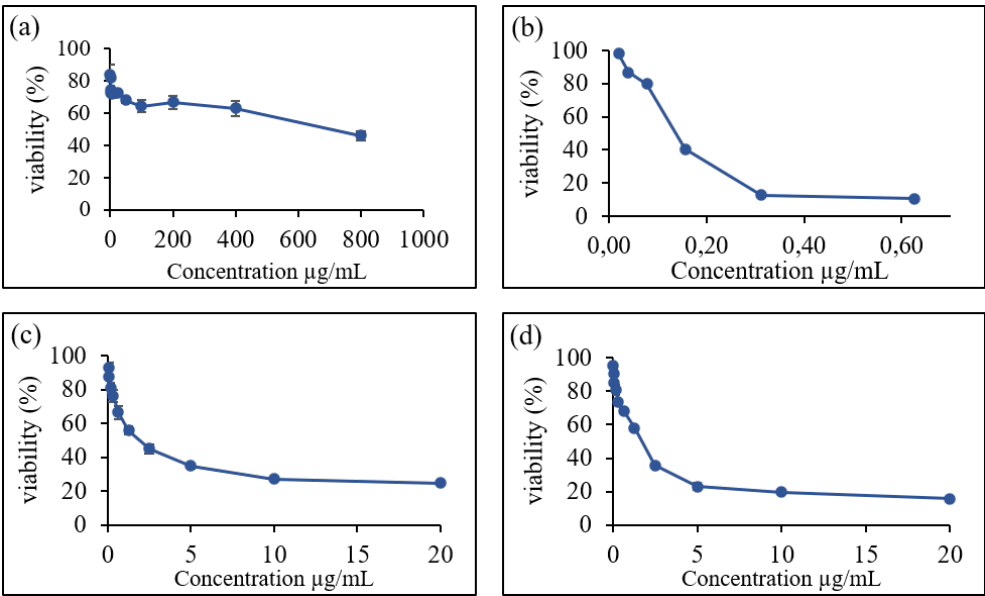


Figure 2. Cytotoxic Effect of the Extract on Human Cancer Cell Lines and Normal Vero Cells. Cell Viability Was Assessed by the MTT Assay After 24 h Exposure to Various Concentrations of the Extract. (a) Vero, (b) T47D, (c) HepG2, and (d) HeLa Cells. Values Are Expressed as Mean ± SD From Three Independent Experiments

findings are consistent with our previous research (Sari et al., 2025). Due to the high selectivity and the low concentrations required to achieve cytotoxicity against cancer cells, the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 is a potential

anticancer drug candidate, especially against T47D, a breast cancer cell line. Two anticancer agents, streptonigrin and oxo-propaline G, were first reported to be produced by *Kitasatospora* sp. MJM383 (Jin et al., 2005).

Table 5. Putatively Identified Metabolites with Reported Anticancer, Antimicrobial, and Antioxidant Activities in the Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7 Based On Mass Spectral Library Matching

Peak	Compound Name	RT (min)	Relative Area (%)	Molecular Formula	Molecular Weight	Match Factor	Reported Biological Activities of Compounds
1	2,4-Di-tert-butylphenol	14.551	3.2	C ₁₄ H ₂₂ O	206.32	728	Antimicrobial, anticancer, and antioxidant (Aravinth et al., 2023 ; Seenivasan et al., 2022 ; Song et al., 2018)
2	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	16.585	2.27	C ₁₆ H ₃₀ O ₄	286.41	692	Antimicrobial, anticancer, analgesic, and antioxidant (Abdulhafiz et al., 2022)
3	Dodecanoic acid, 1-methylethyl ester	17.204	1.1	C ₁₅ H ₃₀ O ₂	242.4	778	No activity reported
4	Benzophenone	17.317	5.85	C ₁₃ H ₁₀ O	182.22	824	Antimicrobial, anticancer, anti-inflammatory, and antiviral (Lei et al., 2024)
5	3-Trifluoroacetoxy - dodecane	18.657	2.08	C ₁₄ H ₂₅ F ₃ O ₂	282.35	736	Antimicrobial and anti-cancer (Hajjar et al., 2017)
6	Methoxyacetic acid, 3-tridecyl ester	18.806	0.74	C ₁₆ H ₃₂ O ₃	272.42	709	No activity reported
7	Cyclopropane - tetradecanoic acid, 2-octyl-, methyl ester	18.922	0.66	C ₂₆ H ₅₀ O ₂	394.67	601	Antimicrobial (Youssef et al., 2023)
8	3-[5-(2-Acetoxypropyl)-2,2-dimethyl-[1,3]dioxolan-4-yl]acrylic acid, methyl ester	18.976	2	C ₁₄ H ₂₂ O ₆	286.32	588	No activity reported
9	Decane, 2,3,5,8-tetramethyl-	19.055	2.27	C ₁₄ H ₃₀	198.39	753	No activity reported
10	Pentanoic acid, 3-methyl-4-oxo-	19.32	0.71	C ₆ H ₁₀ O ₃	130.14	656	Antimicrobial, anticancer, and antiaging (Sahu et al., 2020)
11	1,2-Cyclopentanedi - carboxylic acid, 4-(1,1-dimethylethyl)-, dimethyl ester, (1 α ,2 β ,4 β)-	19.708	0.66	C ₁₃ H ₂₂ O ₄	242.31	621	No activity reported
12	1-Ethynyl-3,trans(1,1-dimethylethyl)-4,cis-	19.772	0.56	C ₁₃ H ₂₂ O ₂	210.31	636	Anticancer, anti-inflammatory, and antioxidant
13	Hexadecane, 4-Methyl-	19.993	0.54	C ₁₇ H ₃₆	240.47	734	Anticancer (Barathi and Raja, 2020)

Peak	Compound Name	RT (min)	Relative Area (%)	Molecular Formula	Molecular Weight	Match Factor	Reported Biological Activities of Compounds
15	9-Octadecenoic Acid, (2-Phenyl-1,3-dioxolan-4-yl)methyl Ester, trans-	20.293	0.90	C ₂₈ H ₄₄ O ₄	444.65	638	Antimicrobial and anti-inflammatory (Rahman et al., 2023)
16	[1,1'-Bicyclopropyl]-2-octanoic Acid, 2'-Hexyl-, Methyl Ester	20.435	0.91	C ₂₁ H ₃₈ O ₂	322.53	622	Antimicrobial (Roslizawaty et al., 2023 ; Sathish et al., 2020)
18	2,8-Decadienedioic Acid, Diethyl Ester	21.027	7.25	C ₁₄ H ₂₂ O ₄	254.32	620	Antimicrobial (Ray et al., 2020)
22	Isoxazolidine-3,5-dicarboxylic Acid, Dimethyl Ester	21.697	0.63	C ₇ H ₁₁ NO ₅	189.17	663	No activity reported
23	d-Lyx-d-manno-nononic-1,4-lactone	21.969	0.27	C ₉ H ₁₆ O ₉	268.22	673	Antimicrobial (Mad Nasir et al., 2021)
24	Tetraacetyl-d-xylonic Nitrile	22.252	1.11	C ₁₃ H ₁₇ NO ₉	343.29	644	Anticancer and antioxidant (Hamad et al., 2022)
25	Phthalic Acid, 5-Methylhex-2-yl Butyl Ester	22.384	2.21	C ₁₉ H ₂₈ O ₄	320.42	790	No activity reported
28	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.435	1.96	C ₁₇ H ₂₄ O ₃	276.37	858	Antimicrobial and anticancer (El-Fayoumy et al., 2021 ; Yuliani et al., 2023)
29	Tetradecane, 2,6,10-Trimethyl-	23.483	2.27	C ₁₇ H ₃₆	240.47	787	Antibacterial and nematocidal (Aisha et al., 2020)
30	Pyrrolo[1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylpropyl)-	23.683	0.83	C ₁₁ H ₁₈ N ₂ O ₂	210.27	689	Antimicrobial, anticancer, and antioxidant (Mangrolia and Osborne, 2020 ; Manimaran and Kannabiran, 2017)
31	Acetic Acid, 2-Oxa-7-thiatricyclo[4.3.1.0(3,8)]-dec-10-yl Ester	23.765	0.68	C ₁₀ H ₁₄ O ₃ S	214.28	646	No activity reported
33	n-Hexadecanoic Acid	24.146	1.22	C ₁₆ H ₃₂ O ₂	256.42	783	Antimicrobial, anticancer, and antioxidant (Sangpairoj et al., 2022 ; Wahyuni et al., 2024)
34	Phthalic Acid, Butyl Undecyl Ester	24.251	2.75	C ₂₃ H ₃₆ O ₄	376.50	634	Antimicrobial, anticancer, and urinary acidulant (Al-Gara'awi et al., 2019 ; Nsofor et al., 2023)
37	5,8,11-Heptadecatriynoic Acid, Methyl Ester	24.915	18.47	C ₁₈ H ₂₄ O ₂	272.40	672	No activity reported
38	Octadecanal	25.323	1.41	C ₁₈ H ₃₆ O	268.48	796	Antimicrobial (Garba et al., 2016)

Peak	Compound Name	RT (min)	Relative Area (%)	Molecular Formula	Molecular Weight	Match Factor	Reported Biological Activities of Compounds
40	Panaxynone	26.108	2.89	C ₁₇ H ₂₂ O	242.36	640	Anticancer and anti-inflammatory (Accogli et al., 2024; Singh and Lamkang, 2022)
41	2-Trifluoroacetoxy - pentadecane	26.476	0.94	C ₁₇ H ₃₁ F ₃ O ₂	324.40	784	No activity reported
42	7-Methyl-Z-tetradecen-1-ol Acetate	26.898	0.35	C ₁₇ H ₃₂ O ₂	268.40	736	Anticancer, anti-inflammatory, and hepatoprotective (Sobhy et al., 2023)
43	2,5-Dimethoxy-4-nitrophenethylamine, N,N-Bis(trimethylsilyl)-	27.459	4.89	C ₁₆ H ₃₀ N ₂ O ₄ Si ₂	370.00	590	No activity reported
44	[5,9-Dimethyl-1-(3-phenyloxiran-2-yl)-deca-4,8-dienylidene]-(2-phenyl-aziridin-1-yl)-amine	27.714	1.18	C ₂₈ H ₃₄ N ₂ O	414.60	630	No activity reported
46	Heneicosane, 11-(1-Ethylpropyl)-	28.224	0.70	C ₂₆ H ₅₄	366.71	766	Antimicrobial, anticancer, and antineoplastic (Subramanian et al., 2020)
50	Glycine, N-(N-Glycyl-L-leucyl)-	28.911	0.49	C ₁₀ H ₁₉ N ₃ O ₄	245.28	709	No activity reported
51	Isoobtusilactone A	29.023	0.89	C ₁₉ H ₃₂ O ₃	308.50	605	Anticancer (Kuo et al., 2007)
52	1-Hexadecanol, 2-methyl-	29.207	0.55	C ₁₇ H ₃₆ O	256.47	711	Antimicrobial and antioxidant (Seenivasan, 2018)
54	Methyl phaseate	29.663	0.41	C ₁₆ H ₂₂ O ₅	294.34	643	No activity reported
55	(4-Isopropylidenebicyclo-[3.2.0]hept-2-en-6-ylidene)acetic Acid, Methyl Ester	29.884	1.76	C ₁₃ H ₁₆ O ₂	204.26	668	No activity reported
57	Perhydrocyclopropa-[e]azulene-4,5,6-triol, 1,1,4,6-tetramethyl	30.571	0.77	C ₁₅ H ₂₆ O ₃	254.36	631	Anti-inflammatory and insecticidal (Kumar et al., 2022)
58	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	30.758	0.60	C ₁₄ H ₁₆ N ₂ O ₂	244.29	682	Antifungal (Sanjenbam and Krishnan, 2016)
59	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	31.795	0.73	C ₂₆ H ₅₄	366.71	745	Anti-inflammatory (Luthfi-ana Hasna et al., 2023; Sophiya et al., 2021)
60	2-Myristoylpantetheine	31.285	0.32	C ₂₅ H ₄₄ N ₂ O ₅ S	484.70	738	Antimicrobial (Amudha et al., 2018; Wang et al., 2018)

Peak	Compound Name	RT (min)	Relative Area (%)	Molecular Formula	Molecular Weight	Match Factor	Reported Biological Activities of Compounds
64	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl]-	34.121	2.40	C ₂₅ H ₃₆ O ₂	368.55	855	No activity reported
65	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E)-	34.458	1.95	C ₂₇ H ₄₄ O ₃	416.646	681	Biocide (Chirumamilla et al., 2022; Shareef et al., 2016)
66	9-(2',2'-Dimethylpropanoil - hydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]fluorene	34.737	0.51	C ₃₀ H ₄₂ Cl ₂ N ₄ O ₃	577.60	743	Antimicrobial, anticancer, and antioxidant (Dewi et al., 2020; Seenivasan, 2018)
67	Nor-diazepam, 3-[[N-hydroxymethyl]amino - carbonyloxy]-	34.798	1.16	C ₁₈ H ₁₆ ClN ₃ O ₄	373.80	623	Antiviral (Sympli, 2021)

3.4 Secondary Metabolite Profile Analysis

The ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 produced at different cultivation periods exhibited variations in color ranging from deep yellow to reddish hues (Figure 3). Secondary metabolite profiling using thin-layer chromatography (TLC) with a chloroform: ethyl acetate:methanol (6:3:1) solvent system revealed three well-resolved spots. All four extracts (Figure 3b) showed identical secondary metabolite profiles when observed under UV light at 254 nm and 366 nm, as well as under visible light. At 254 nm, a single spot was detected with an R_f of 0.73; at 366 nm, three spots were observed with R_f values of 0.73, 0.61, and 0.46. Thus, despite the differences in extract colouration, all samples contained the same secondary metabolites.

Although the crude extracts displayed slight variations in color across fermentation batches, the TLC chromatograms consistently showed the same number of major spots with comparable R_f values. These findings suggest that the principal metabolites remained unchanged, whereas color variation may result from differences in pigment concentration, oxidation processes, or minor metabolites present at levels below the detection limit of TLC. Pigment production by actinomycetes during fermentation is influenced by a complex interplay of physicochemical and nutritional factors (Priya, 2026).

Temperature, pH, salinity, composition of the growth medium, incubation time, and agitation speed are critical parameters that play a crucial role in determining pigment colour and yield (Sayed et al., 2023; Silva-Arias et al., 2025; Wan et al., 2023). With optimal conditions varying by species, optimizing all parameters is needed to achieve optimal pigment and secondary metabolite production.

3.5 GC-MS Analysis

GC-MS analysis tentatively identified 47 volatile compounds in the ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7, of which 26 possess antimicrobial, antioxidant, and anticancer activity, as described in Table 5. The major constituents included 5,8,11-heptadecatriynoic acid, methyl ester (18.47%), 2,8-decadienedioic acid, diethyl ester (7.25%), benzophenone (5.85%), 2,4-di-tert-butylphenol (3.20%), and panaxynone - (2.89%), as shown in Figure 4. Several compounds possess antimicrobial, antioxidant, and anticancer activity, including fatty acid esters, alkanes, decane, phenols, and carboxylic acids. Among these compounds, 2,4-di-tert-butylphenol is one of the most extensively reported microbial metabolites and has demonstrated broad-spectrum antibacterial, antioxidant, and cytotoxic activities through disruption of microbial membranes, free radical scavenging, and induction of apoptosis in cancer cells (Fuqiang et al., 2020).

In addition, diketopiperazines identified in the extract, namely Pyrrolo[1,2-a]pyrazine-1,4-dione derivatives, are common secondary metabolites produced by actinomycetes and have been reported to possess antibacterial, antibiofilm, antioxidant, and cytotoxic properties, suggesting that these metabolites may contribute to the biological activities observed in the present study (Huang et al., 2010; Mangrolia and Osborne, 2020; Siddharth et al., 2021). Benzophenone and 2,4-di-tert-butylphenol, a ketone and a phenol, were reported to have anticancer and antimicrobial activity, with 5.85% and 3.2% content, respectively (Lei et al., 2024; Seenivasan, 2018).

The combined presence of phenolic compounds, diketopiperazines, fatty acids, and aromatic metabolites may explain the potent antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*, as well as the strong

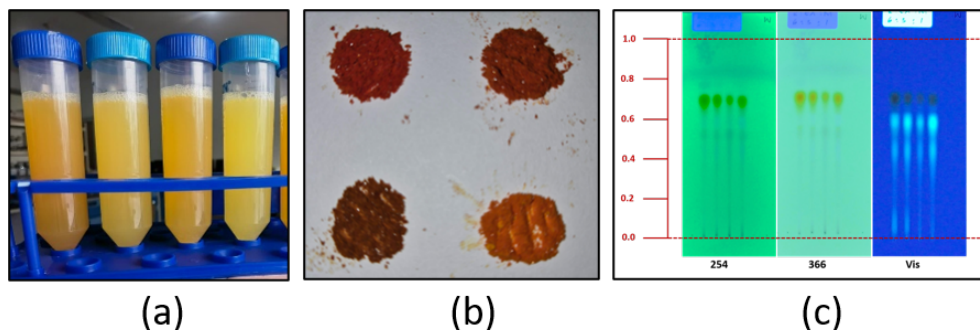


Figure 3. a) Crude fermentation of *Kitasatospora* sp. TC-ARCL7, b) Ethyl Acetate Crude Extract, c) TLC

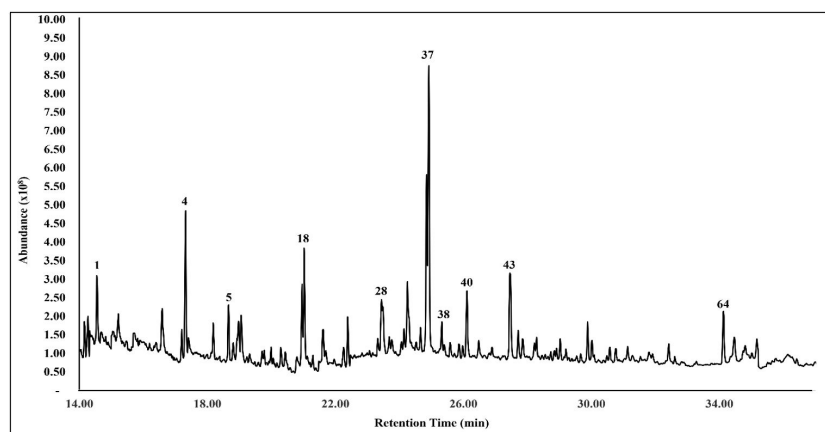


Figure 4. Total Ion Chromatogram (TIC) of the Ethyl Acetate Extract of *Kitasatospora* sp. TC-ARCL7 Obtained by GC-MS Analysis

cytotoxicity against T47D, HepG2, and HeLa cells. As GC-MS detects only volatile and thermally stable compounds, the observed activities likely result from synergistic interactions among multiple metabolites, including undetected non-volatile compounds such as polyketides and non-ribosomal peptides. However, the biological activities of the identified compounds were inferred from previous reports and remain to be confirmed through bioactivity-guided isolation and structural characterization.

4. CONCLUSIONS

The ethyl acetate extract of *Kitasatospora* sp. TC-ARCL7 exhibited selective cytotoxicity against T47D, HeLa, and HepG2 cell lines, with the strongest activity against T47D cells ($IC_{50} = 0.20 \pm 0.04 \mu\text{g/mL}$). The extract also demonstrated antimicrobial activity against *Bacillus subtilis* and *Escherichia coli* ($MIC = <1.22 \times 10^{-3} \text{ mg/mL}$), as well as *Staphylococcus aureus* ($MIC = 4.88 \times 10^{-3} \text{ mg/mL}$). The contrasting antioxidant responses, characterized by strong FRAP activity ($118.70 \pm 18.8 \text{ mM Fe(II)/g}$) and weak DPPH radical-scavenging activity ($IC_{50} = 513.35 \mu\text{g/mL}$), suggest that the extract's antioxidant capacity may be mediated predominantly by electron-transfer processes

rather than direct free-radical quenching. This study is limited to evaluating crude extracts, and the active compounds responsible for each biological activity remain unidentified. Further purification and structural elucidation are required. The observed biological activities may be associated with the detected secondary metabolites; however, this relationship remains tentative and requires confirmation through purification of active compounds and bioactivity-guided fractionation. This result demonstrates promising in vitro cytotoxic activity and warrants further investigation through bioassay-guided isolation, mechanism-of-action studies, and in vivo evaluation.

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