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Rhizospheric actinomycetes from turmeric (*Curcuma longa* L.): Isolation, detection of the biosynthetic gene clusters, and anticancer activity against T47D cancer cells

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ABSTRACT

This study investigates actinomycete bacteria in the rhizosphere of turmeric rhizomes, which can produce bioactive compounds like their host, particularly those with anticancer properties. The main goals were to isolate these bacteria, analyze their biosynthetic gene clusters (BGCs), and test their effects on T47D cancer cells. We identified seven isolates, revealing three BGC combinations of PKS1, PKS2, and non-ribosomal peptide synthetase. Notably, isolate TC-ARCL7, which had both PKS1 and PKS2 genes, demonstrated significant anticancer activity against T47D cells, with an IC50 of 0.2 µg/ml, much more potent than Doxorubicin (7.9 µg/ml), curcumin (23.13 µg/ml), and turmeric ethanol extract (50 µg/ml). This isolate was closely related to *Kitasatospora misakiensis* or *Kitasatospora purpeofusca*, with 99.08% sequence similarity. The findings highlight that similar BGCs do not always correlate with anticancer activity and suggest the potential for developing new pharmaceutical compounds.

INTRODUCTION

Research is underway on turmeric (*Curcuma longa* L.) for potential new drug development [1,2], but high production costs limit its application. As a result, scientists are investigating beneficial microbes found in plants and soil [3,4], particularly actinomycetes, which produce about 10,000 of the 23,000 known bioactive compounds [5], offering many potential health benefits [6]. Actinomycetes thrive in various conditions, increasing the likelihood of discovering new compounds. A study by Sapkota *et al.* [7] found certain soil actinomycetes to be 90% effective with minimal risk to commercial products.

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Research indicates that actinomycetes from plant roots share properties with their host, including anticancer [4,8], antibacterial [9,10], antifungal, immunosuppressive, and antiparasitic effects. These benefits stem from bioactive compounds produced by biosynthetic gene clusters (BGCs), which include critical components like non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) [11]. The PKS gene synthesizes polyketide (PK) chains from acyl-CoA, while the NRPS gene synthesizes peptide chains from amino acids [12]. PK and non-ribosomal peptides (NRP) are the primary secondary metabolites of the genus *Streptomyces*, with nearly three-quarters of its BGCs containing PKS and NRPS genes. Detecting BGCs encoding PKS1, PKS2, and NRPS provides a promising method for predicting secondary metabolites or natural products synthesized through these pathways [13].

Previous research found anticancer activity in rhizospheric actinomycetes from potential anticancer plants [4,8] but did not investigate their BGCs. Despite limitations in current research,

Figure 1. Turmeric (*Curcuma longa* L.) plant.

studying the PKS-NRPS genes of turmeric's actinomycetes could uncover valuable bioactive compounds for cancer treatment. This study focuses on isolating turmeric rhizosphere actinomycetes, detecting their NRPS, PKS1, and PKS2 gene content, and *in vitro* testing of the isolate's extract on aggressive T47D cancer cells.

MATERIAL AND METHODS

Materials

Turmeric plantation soil samples were collected from Gemantar village, Jumantono, Karanganyar District, Central Java, Indonesia, at 7.6778°S, 111.0311°E, and 458 masl in May 2023. The soil was dug from 3 to 5 cm deep, about 0–10 cm away from approximately seven-month-old turmeric plants (Fig. 1) until the rhizomes were exposed [14]. About 5 g of rhizosphere soil were collected using a sterile spatula and placed into a sterile flask under sterile conditions. Before further analysis, the soil samples were stored at 4°C.

Rhizosphere actinomycetes of turmeric

Isolation and purification

Starch Casein Agar (SCA) and yeast malt agar (ISP2) were used as growth media, with their composition and preparation following the guidelines by Shirling and Gottlieb [15]. The isolation steps were adapted from Osama *et al.* [16]. The media were weighed according to the reference, sterilized in an autoclave at 121°C for 15 minutes, and then supplemented with 100,000 IU of nystatin (1 ml/l) to inhibit fungal growth. One gram of soil sample was mixed with 9 ml of sterile 0.9% NaCl, vortexed for 2 minutes, and filtered through filter paper. The solution was diluted from 10-¹ to 10-6. Actinomycetes were isolated using the pour plate method, where 1 ml from each dilution was added to Petri dishes containing 15 ml of SCA medium at approximately 40°C, allowed to solidify, and then incubated at 28V–30°C for 7–21 days. The streak plate method was subsequently used to transfer actinomycetes colonies with distinct characteristics to fresh SCA media, each labeled with a TC-ARCLx code. A single colony was purified on ISP2 slants (pH 8.0 and salinity 2 mg/l), and the pure actinomycetes isolates were stored in an starch casein broth (SCB) medium with 15% glycerol at −80°C for long-term preservation.

Morphology characterization

The morphology of individual actinomycetes colonies was visually examined using a stereomicroscope. Key morphological features assessed included colony form, height, edges, and consistency, focusing on the color of the aerial mycelium (in adult culture), substrate mycelium, and pigment type. The color analysis was based on the RHS Color Chart [17].

DNA isolation

The DNA isolation stages of actinomycetes were adapted from Rante *et al.* [10] using DNeasy PowerLyzer Microbial Extraction Kit (Qiagen) [18] with some modifications. A total of 1.8 ml of a 4-day-old liquid culture was placed in a collection tube and centrifuged at 10,000 rpm for 30 seconds. The pellet was then resuspended in 300 µl of powerbead solution and incubated in a shaker incubator at 65°C for 10 minutes to enhance DNA yield. The extraction was then continued according to the kit procedure until the genome was obtained. DNA concentration and purity were assessed using a Multiskan Sky-high microplate spectrophotometer at 260 and 280 nm wavelengths. Purity was determined by calculating the optical density (OD) ratio at these wavelengths (OD260/OD280). High DNA purity is indicated by a ratio valued between 1.8 and 2.0. A ratio below 1.8 suggests contamination from large molecular compounds, such as proteins, while a ratio above 2.0 indicates contamination from small molecular compounds, such as RNA.

Detection of PKS1, PKS2, and NRPS biosynthetic genes

Amplification of the PKS1 gene segment was performed using degenerate primers KS-F (5′-CCSC AGSAGCGCSTSYTSCTSGA-3′) and KS-R (5′-GTSCCS GTSCCGTGSGYST CSA-3′). The PKS2 gene fragment was amplified with primers KS∞ (5′-TSGCSTGCTTG GAYGCSATC-3′) and KSβ (5′-TGGAANCCGCCG AABCCTCT-3′) [19]. For NRPS, degenerate primers A3F (5′-GCSTACSYSATSTACACSTCSGG-3′) and A7R (5′-SASGT CVCCSGTSCGGTAS-3′) were used [20].

Each polymerase chain reaction (PCR) series included a negative control without a DNA template [21]. The PCR mix (25 µl) consisted of 2.5 µl DNA template, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 12.5 µl HS Taq RedMix (2x), and 8 µl nuclease free water (NFW) [22]. The PCR conditions consist of an initial denaturation at 95°C for 1 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing for 15 seconds at 55°C, 57°C, and 59°C for NRPS, PKS1, and PKS2 primers, respectively, extension at 72°C for 15 seconds, and a final extension at 72°C for 7 minutes [23].

Secondary metabolite production and extraction

The actinomycete starter cultures were grown in 100 ml of SCB medium and incubated at 28°C–30°C for 3 days at 170 rpm. The cultures were transferred to a 1-l Erlenmeyer flask containing 200 ml of SCB medium, with a 1% inoculum, and then incubated at 28°C–30°C for 10 days at 170 rpm. The actinomycete cultures were centrifuged at 6,000 rpm for

10 minutes at 4°C. The supernatant was then extracted using liquid-liquid extraction with an equal volume of ethyl acetate for 24 hours in an orbital shaker. The phases were separated with a separating funnel, and the upper layer of ethyl acetate was collected in a porcelain cup and evaporated at 40°C to obtain the extract [5].

Cytotoxicity assay

The cytotoxicity of the ethyl acetate extract from actinomycetes was evaluated on T47D and Vero cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [24]. The cell lines were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were then seeded into 96-well plates and treated with varying concentrations of the ethyl acetate extract dissolved in Dimethyl Sulfoxide. The plates were then incubated at 37°C, 95% humidity, and 5% CO_2 for 24 hours. After incubation, 1% of MTT solution was added and further incubated for 3 hours, followed by the addition of 10% SDS to stop the reaction. The absorbance of the resulting formazan crystals was measured at 595 nm using a Multiscan Sky-high microplate spectrophotometer, and the percentage of cell viability was calculated [25]. The cytotoxicity test was also conducted on hydrochloride doxorubicin (intravenous solution 2 mg/ml, Global Onkolab Farma), which is currently used as a drug, as well as on curcumin (Sigma for synthesis, Merck 20354) and a 96% ethanol extract of turmeric as the host plant of the rhizosphere actinomycete isolate.

Molecular identification with the 16S rRNA gene

Isolates that demonstrated cytotoxic activity against T47D cancer cells were identified by amplifying the 16S rRNA Gene using the PCR method. The procedure employed HS Taq RedMix (2x) according to the specified protocol, utilizing primers 27F (5′-AGAGTTTGATCC TGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [10]. The reaction mixture was prepared to a total volume of 50 µl, comprising 1 µl of DNA template (approximately > 10 ng), 2 µl of 10 nM forward primer, 2 µl of 10 nM reverse primer, 25 µl of HS Taq RedMix (2x), and 20 µl of NFW. The PCR conditions consist of an initial denaturation at 98°C for 2 minutes, followed by 29 cycles of denaturation at 98°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 15 seconds, and a final extension at 72°C for 7 minutes.

Following amplification, the PCR products were visualized by electrophoresis on a 1% agarose gel dissolved in Tris/Borate/EDTA Buffer and 2 µl of SYBR Safe stain, running for 50 minutes at 70V. The results were observed using the Gel Documentation System Alliance™ Q9 Manual™. The completed PCR products were then sent to 1st BASE Malaysia for sequencing via Sanger DNA Sequencing with Capillary Electrophoresis.

Data analysis

A descriptive analysis of the morphological data was conducted by Bergey's Manual of Systematic Bacteriology [26]. The IC_{50} value was calculated using Microsoft Excel with a linear regression formula. Sequencing results were analyzed with the Bioedit program, and comparisons were made against the NCBI GenBank database using BLAST (http://www.ncbi.nih.gov/Blast).

RESULTS AND DISCUSSION

Rizhosphere actinomycetes of turmeric

Actinomycetes were initially isolated on SCA and subsequently purified using an ISP2 medium. Twelve isolates were obtained from the rhizosphere soil of turmeric, each exhibiting unique morphological characteristics. However, effective purification was successful for only seven of these isolates, as the other five remained tightly associated with other bacteria during the growth phase. The seven isolates were then subjected to morphological characterization (Table 1 and Fig. 2).

The actinomycetes colonies from the turmeric rhizosphere displayed a circular morphology. In ISP2 medium, the colors of the aerial mycelium varied from white to grey, while the substrate mycelium ranged from yellow to brown. As illustrated in Table 1 and Figure 2, these actinomycetes produced pigments in a spectrum from light yellow to brownish orange.

Table 1. Morphological characteristics of rhizosphere turmeric actinomycetes isolates in ISP2 media.

Characteristics	Isolate						
	TC-ARCL3	TC-ARCL7	TC-ARCL8a	TC-ARCL12	TC-ARCL16	TC-ARCL17	TC-ARCL19
Colony form	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colony elevation	Convex	Convex	Umbonate	Pulvinate	Pulvinate	Umbonate	Convex
Colony edge	Erose	Erose	Lobate	Undulate	Erose	Erose	Entire
Consistency	Powdery	Powdery	Leathery	Cottony	Cottony	Leathery	Leathery
Aerial mycelium color	Yellowish white	Bluish white	Yellowish white	White	White	White	Yellowish grey
Substrate mycelium color	Strong orange yellow	Strong orange yellow	Moderate orange yellow	Moderate brown	Brownish orange	Pale yellow	Moderate yellow
Diffuse pigment type	Water-soluble pigment	water-soluble pigment	Fat-soluble pigment	Water-soluble pigment	Fat-soluble pigment	Fat-soluble pigment	Fat-soluble pigment
Pigment color	Moderate yellow	Strong orange yellow	Yellowish white	Brownish orange	White	White	Yellowish grey
Gram reaction	Positive	Positive	Positive	Positive	Positive	Positive	Positive

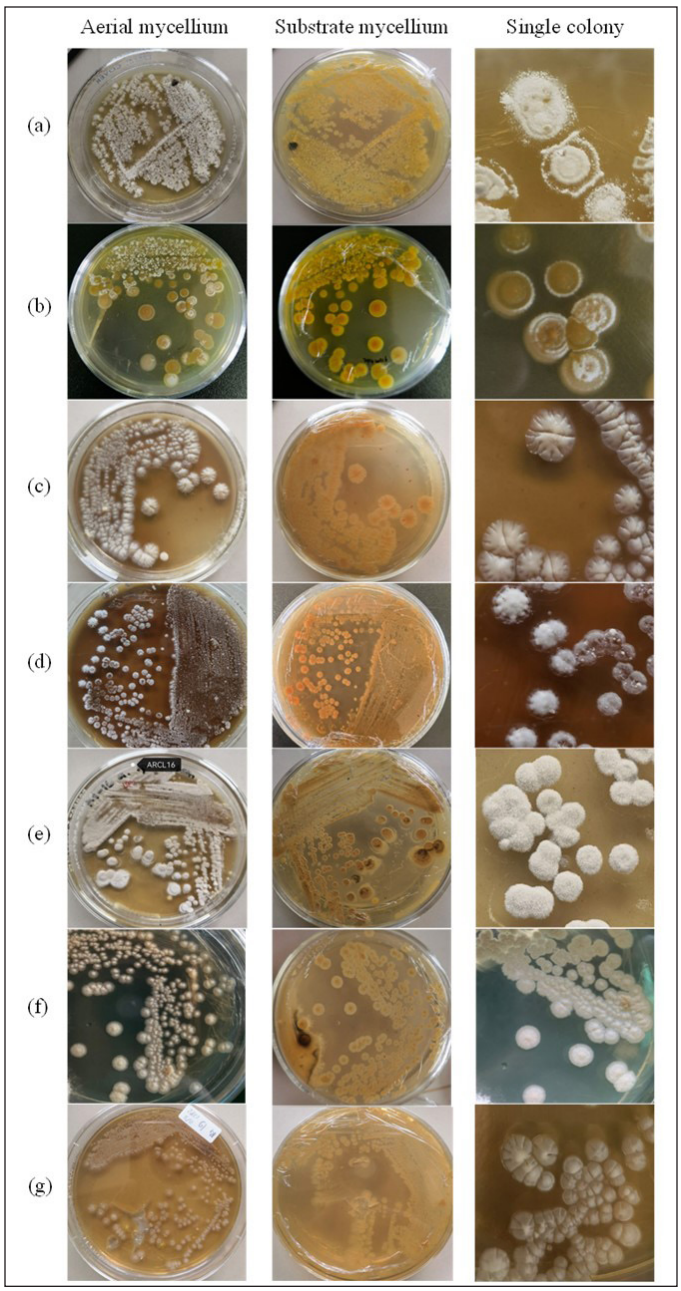

Figure 2. Actinomycetes colony isolated with ISP2 medium. (a) TC-ARCL3, (b) TC-ARCL7, (c) TC-ARCL8a, (d) TC-ARCL12, (e) TC-ARCL16, (f) TC-ARCL17, and (g) TC-ARCL19.

Rante *et al.* [10] reference a "colour wheel" that demonstrates the range of colors that can be produced by substrate and aerial mycelium. The pigments and colors of the mycelium are substrate-dependent, reflecting their essential role in nutrient absorption [27]. Furthermore, the concentration of hydrogen ions in the medium may influence the color of these pigments [28].

Detection of NRPS/PKS genes

This study identified seven actinomycete isolates with at least one BGC gene marker (Table 2 and Fig. 3). The BGC gene markers detected were PKS1, PKS2, and NRPS, measuring 700–800, 554, and 1,200–1,400 bp in size, respectively. These findings align with those of Peng *et al.* [29], who successfully isolated actinomycetes from the rhizosphere of *Panax notoginseng* [29].

Among the seven isolates, three combinations of BGC content were observed: TC-ARCL3, TC-ARCL8a, TC-ARCL16, and TC-ARCL17 contained PKS1, PKS2, and NRPS genes; TC-ARCL7 and TC-ARCL19 had only PKS1 and PKS2; and TC-ARCL12 contained PKS2 and NRPS. According to Safari *et al.* [30], these isolates have the potential to produce various bioactive compounds from the non-ribosomal peptide and PK families due to the presence of KS and A domains in the PKS and NRPS gene clusters [30]. The core domain determines the addition of peptide units, while optional domains modify the peptide or ketide backbone [31]. Doxorubicin, an anthracycline chemotherapy agent currently used in the medical field, is produced from the PKS2 gene cluster of *Streptomyces peucetius varietas caesius* [6,13,32]. These results show that data from genome sequences pointing to secondary metabolite BGCs is essential for discovering new compounds and designing them using synthetic biology. Specifically, PK and NRP can be re-engineered with modular enzymes that interact with specific CoAs or amino acids [33].

Cytotoxicity assay

A widely used cell line in cancer research is T47D, a human breast cancer cell line. T47D cells are a great model for studying the specific effects of progesterone in luminal A subtype breast cancer [34]. This study successfully extracted secondary metabolites from five of the seven isolates using ethyl acetate as the solvent, followed by cytotoxicity tests. The IC_{50} values for T47D cancer cells and Vero cells are detailed in Table 3. Based on Nordin *et al.* [35], the cytotoxicity analysis of the T47D cell line categorized the ethyl acetate extracts into two groups: high (TC-ARCL7) and weak (TC-ARCL12, TC-ARCL16, TC-ARCL17, and TC-ARCL19). In contrast, all isolates showed weak inhibition of Vero cell proliferation.

Among the extracts, only TC-ARCL7 demonstrated a high cytotoxic effect against the T47D cell line, with an IC_{50} value of 0.2 ± 0.02 µg/ml, while showing weak toxicity toward Vero cells, which had an IC_{50} value of 416.04 ± 0.49 µg/ml, so resulted in a selectivity index 2,080.20. A selectivity index of ≥10 is considered promising and indicates strong potential for further research [36,37]. Notably, the cytotoxic efficacy of the TC-ARCL7 extract against T47D cells was 39.5 times lower than hydrochloride doxorubicin (IC_{50} = 7.9 µg/ml), 115.65 times lower than curcumin (IC_{50} = 23.13 µg/ml), and 250 times lower than turmeric ethanol extract (IC_{50} = 50 µg/ml). The cytotoxic activity of ARCL7 ethyl acetate extract against T47D cells is comparable to Doxorubicin from Ebewe Pharma, Unterach, Austria (IC_{50} of 1.845 µg/ml) [32], and Doxorubicin from Sigma, Germany (IC_{50} = 0.2 ± 0.04 µg/ml) [38]. The IC_{50} value of Doxorubicin can vary significantly in different literature due to factors such as cell line conditions, formulation type, and brand.

Considering that Doxorubicin causes various side effects [32,39], these results suggest that TC-ARCL7 could

Table 2. BGC (NRPS/PKS1/PKS2) of rhizosphere turmeric actinomycetes.

BGCs	Isolate						
	TC-ARCL3	TC-ARCL7	TC-ARCL8a	TC-ARCL12	TC-ARCL16	TC-ARCL17	TC-ARCL19
PKS1	+	+	+	-	+	+	+
PKS2	+	+	+	+	+	+	+
NRPS	+	-	+	+	+	+	-

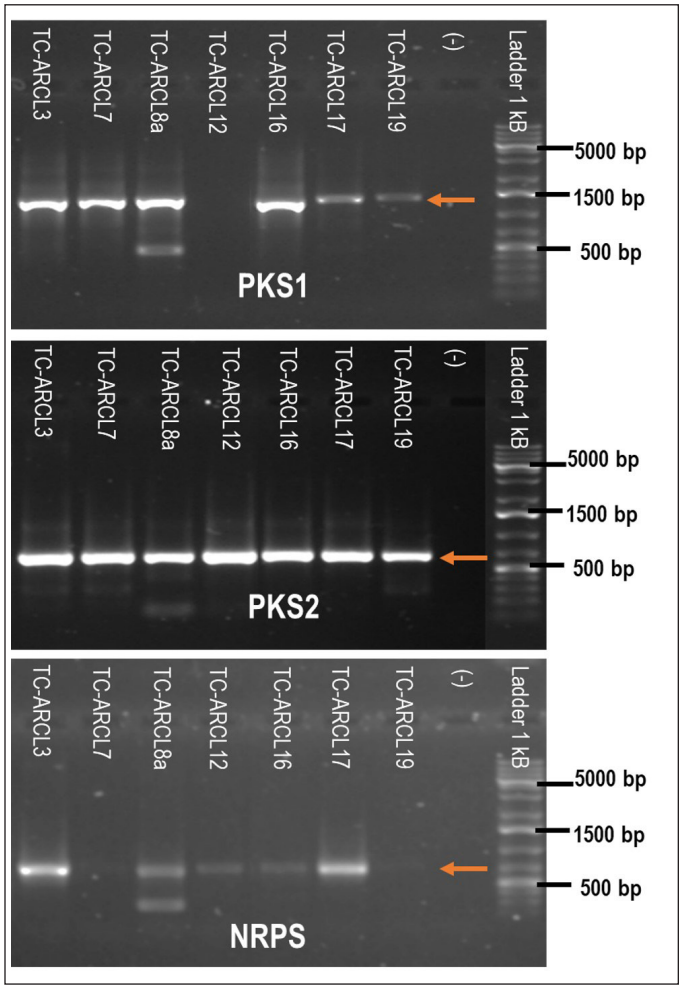

Figure 3. Electropherogram of amplified PKS1, PKS2, and NRPS genes from actinomycetes isolates.

serve as a valuable source for developing new anticancer drugs, given its high toxicity to T47D cancer cells coupled with low toxicity to Vero cells *in vitro*.

Molecular identification of potential isolate

The TC-ARCL7 isolate was identified through molecular amplification of the 16S rRNA biomarker gene. The 16S rRNA gene is favoured for this purpose due to its multicopy nature (150–300 copies in the bacterial genome) and high level of conservation. Its variable regions allow for effective differentiation among species, while its lack of horizontal gene transfer contributes to a slower evolutionary rate [40].

Table 3. IC_{50} value of ethyl acetate extract of turmeric rhizosphere actinomycetes on T47D cancer cells and vero cells.

Sample	IC_{50} on T47D (µg/ml)	IC_{50} on vero (µg/ml)	Selectivity index
TC-ARCL7	0.2 ± 0.02	416.04 ± 0.49	2,080.20
TC-ARCL12	226.78 ± 16.06	377.34 ± 17.22	1.66
TC-ARCL16	102.04 ± 8.23	275.96 ± 16.32	2.70
TC-ARCL17	128.50 ± 8.30	482.4 ± 19.63	3.51
TC-ARCL19	207.09 ± 2.34	223.7 ± 12.6	1.08
Curcumin	23.13 ± 1.67	55.16 ± 4.19	2.38
Turmeric ethanol extract	50.00 ± 0.7	68.10 ± 11.00	1.36
Doxorubicin	7.9 ± 0.23	109.82 ± 9.65	13.90

Nucleotide sequences from the forward and reverse reads were assembled into contigs and were compared to the GenBank database using BLAST. The TC-ARCL7 isolate exhibited a sequence similarity of 99.08% with *Kitasatospora misakiensis* (NBRC 12891, NR 112321.1) and *Kitasatospora purpeofusca* (NBRC 12905, NR 1123330). A similarity percentage between 98.7% and 99% indicates species delineation [41]. Therefore, this finding implies that the actinomycetes isolate belongs to the same species as these identified organisms. The GenBank accession number for TC-ARCL7 is PP212015.

The genus *Kitasatospora* is notable for its ability to produce a wide range of bioactive compounds, with over 50 known secondary metabolites exhibiting various biological activities, including antitumor, immunomodulatory, antiviral, herbicidal, and antiprotozoal effects [42]. Research by Li *et al.* [43] further indicates that *Kitasatospora* has a high diversity of secondary metabolite biosynthetic gene clusters distinguishing it from the closely related genus *Streptomyces* [43].

CONCLUSION

This study reveals that various microbes, including actinomycetes, thrive in the rhizosphere of turmeric, with seven isolates identified that feature three combinations of BGCs. The presence of specific BGCs does not necessarily correlate with anticancer activity. Notably, only one extract—the TC-ARCL7 ethyl acetate extract—exhibited significant anticancer effects against T47D cancer cells. This isolate was closely related to *K. misakiensis* NBRC 12891 (NR 112321.1) and *K. purpeofusca* NBRC 12905 (NR 1123330). Further research, such as optimization of growth conditions to obtain various extracts and purifications of bioactive metabolites, is needed to

gather comprehensive data that can assist in the discovery of new cancer treatments.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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