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Chemical profile, Antimicrobial and Antitumor Activities of *Streptomyces rochei* SUN35 Strain

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Abstract

The objective of the research is to synthesize anticancer metabolites from a novel isolated strain of *Streptomyces* sp. from cultivated soil. In addition, the chemical characterization and identification of the produced active metabolite has been studied. The antimicrobial and cytotoxic potencies of EtOAc extract of *S. rochei* were evaluated. The extract inhibited the growth of *Staphylococcus aureus, S. aureus (*MRSA), *Pseudomonas aeruginosa*, *Aspergillus fumigatus, Fusarium solani* and *Penicillium chrysogenum*with inhibition zones (mm) of 16, 16, 17, 16, 22, 23, respectively. Furthermore, the extract exhibited noteworthy cytotoxic properties against the hepatoblastoma cell line (HepG2) and human epithelial cell line (Caco-2) with IC50 values of 818.10 µg/ml and 572.50 µg/ml, respectively. Based on the 16S rRNA sequencing, the bacterial strain was recognized as *S. rochei* both phenotypically and genotypically. It was then entered into the GenBank database under the accession number OR492299. The primary GC-MS chemical fingerprint of the EtOAc extract was characterized. Nineteen compounds were found, with relative amounts of 73.43, 5.61, and 3.13%, classified as the principal ingredients. These compounds included 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, 13-docosenamide, (Z)-, and 9-octadecenamide, (Z). These results suggested that *S. rochei*EtOAc extract could be a valuable source of anticancer agents.

Keywords:*Streptomyces rochei*, antimicrobial activity, Genotypic identification, GC-MS, Anticancer metabolites.

1. Introduction

Despite the availability of numerous therapeutic approaches for the treatment of cancer, it is still one of the main causes of death and morbidity in the world. The World Health Organization (WHO) forecasted about 10 million fatalities in 2020; by 2030, it is predicted that there will be 21.6 million instances of cancer worldwide. These predictions are based on data from 2022 [1]. The search for therapeutics with lower side effects and/or higher therapeutic efficacy against resistant malignancies has grown due to the high toxicity and adverse impacts of chemotherapy treatments [2]. Finding new chemotherapy drugs is a top priority, with actinomycetes being one of the main sources of biologically active metabolites. *Streptomyces* species are filamentous, spore-forming, Gram-positive bacteria that are members of the Actinobacteria phylum. It has the capacity to generate a wide range of secondary metabolites, each of which has unique biological properties and the potential to be used as therapeutics. The most abundant natural product source is *Streptomyces* strains, particularly for therapeutically relevant antibiotics and anticancer metabolites [3].

Approximately 100,000 antibiotics and anticancer metabolites are produced by *Streptomyces*, and these make up 70–80% of all naturally occurring bioactive compounds with pharmacological uses [4]. These recognized anticancer substances made by *Streptomyces* species have been incorporated into

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chemotherapy regimens for people. Aureolic acids (mithramycin), anthracyclines (aclarubicin, daunomycin, and doxorubicin), enediynes (neocarzinostatin), non-ribosomal peptides (bleomycin and actinomycin D), antimetabolites (pentostatin), indolocarbazoles, isoprenoides, macrolides, mitomycin C, and epoxomicin represent a number of the structural classes these metabolites belong to [5-11].

For drug screening programs, it was therefore thought to concentrate on the effective isolation of novel Streptomyces species from terrestrial sources. Each genome of Streptomyces has many clusters of biosynthetic genes (BGCs), which are the source of a wide range of bioactive chemicals used in agriculture or medicine [12,13]. Actinobacteria are thought to be poorly studied in Egyptian soil habitat, with very few studies being published [14,15,16,17].

From the above perspectives, the current investigation sought to evaluate the antimicrobial and anticancer activity of the extracted metabolites of *S. rochei* SUN35. Consequently, the further GC-MS chemical profile of the extracted metabolites was studied.

2. Material & methods

2.1. Source of *Streptomyces* SUN35

*S. rochei*was isolated from agricultural soil by utilizing the dilution plate technique [18], the starch casein agar (SCA) treated with Nystatin (50 mg/ml) and Rifampicin (10 mg/ml) to avoid any fungal or bacterial contamination [3].

2.2. Identification of *S. rochei* via phenotypic and genotypic characterization

Using various growth media, including ISP1 (Tryptone-Yeast Extract Agar), ISP2 (Yeast Extract-Malt Extract Agar), ISP3 (Oatmeal Agar), ISP4 (Inorganic Salts-Starch Agar), ISP5 (Glycerol-Asparagine Agar), and ISP7 (Tyrosine Agar), the growth capacity, pigment production, and color of both aerial and substrate mycelium were assessed [18]. By comparing the color with chips from the ISCC-NBS centroid color charts, the color was visually identified [19]. Using direct microscopical examination, the types of hyphae containing spores and their chain morphology were identified, and a scanning electron microscope (SEM) was used to analyze the spore surface's shape. *S. rochei* was examined for production of catalase, urease, hydrogen sulfide, nitrate reductase, starch hydrolysis, casein hydrolysis. Subsequently, L-tryptophane, L-tyrosine, and L-asparagine were used as nitrogen sources. Additionally, the utilization of carbon sources was done in accordance with the modified Pridham and Gottlieb [18] method utilizing ISP 9 and the addition of each of the following sugars separately: cellulose, sucrose, lactose, fructose and D-glucose (positive control).

The Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) was used to extract DNA. The reconstituted cells were mixed with the appropriate amount of Bashing Bead Buffer. To promote cell lysis, proteinase K was added, and the cells were incubated at 65°C for one hour. The universal bacterial primer sets 27f: 5'AGAGTTTGATCMTGGCTCAG3' and 1522r: 5'AAGGAGGTGATCCANCCRCA3' which utilizing genomic DNA as a template. 10 mMdNTPs, 20 mg/ml BSA, 10 pmol of each primer, 1u Phire Hot Start II DNA Polymerase, and Phire Green Hot Start II DNA Polymerase were used for all PCRs. The conditions for amplification were those given by Trujillo [20]. The thermocycling conditions were as follows: 30 seconds of initial denaturation at 98°C was followed by 35 cycles of denaturation at 98°C for 5 seconds, annealing for 5 seconds, and 12 seconds of extension at 72°C, with a final extension lasting 1 minute at 72°C.

The nucleotide sequence that resulted was entered into the GenBank database with the accession number OR492299 and compared to the sequences that had been recorded in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). MEGA 7 software was used to create the phylogenetic tree [21].

2.3. Extraction of Secondary metabolites

A total of 30 ml of starch casein (SC) liquid medium was inoculated with *S. rochei*, and the mixture was then cultured for 7 days at 100 rpm and 28 ± 2 °C. Three times in succession, the culture was extracted using equal volumes of ethyl acetate while being shaken vigorously for 30 minutes. To achieve a final concentration of 100 mg/ml for antimicrobial screening, fractions of ethyl acetate have been redissolved in ethanol after being vacuum evaporated into vials that have been previously weighed.

2.4. Antimicrobial screening

The standard well diffusion test was used to assess the antibacterial activity of the *S. rochei*EtOAc extract [22].Crude metabolic extracts with concentration 100 mg/ml were tested against two Gram-positive bacteria (*Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* "MRSA") and one Gram-negative bacteria (*Pseudomonas aeruginosa*).The Tested strain was

compared to Amoxicillin/Clavulanic acid (30µg) antibiotic as a standard control. The antifungal activity was tested against three pathogenic fungal cultures: *Aspergillus fumigatus*, *Fusarium solani* and *Penicillium chrysogenum*. Following the incubation period, the diameter of the growth inhibition zones was precisely measured in millimeters.

2.5. Cytotoxic activity

Measurement of potential tumor cytotoxicity of the metabolic extracts was investigated using an MTT assay according to Mosmann [23] at Vacsera Center Cairo, Egypt. Briefly, 96-well plates containing 104 cells per well were plated with 200 µl PBS for 48 hours prior to extract treatment to achieve 80% confluence in the colon Caco-2 and liver HepG2 and human cell lines. Subsequently, two cell lines were cultured for 48 hours in RPMI-1640, containing 200, 400, 600, 800, and 1000 µg/ml of the *S. Rochei* EtOAc extract, in place of the previous culture media. 20 µl of MTT solution (3, 4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide was added to each well and incubated for 4 h in 37°C in dark. To dissolve the formazan crystals that had formed, 200 µl of acidic isopropanol was added to the supernatant, mixed thoroughly, and then incubated for a further hour at 37°C in the dark. Using an ELISA reader, the absorbance at 570 nm was measured.

2.6. Gas Chromatography-Mass Spectrometry (GC-MS)

The *S. rochei* OR492299 EtOAc extract's chemical constituents were determined by GC-MS analysis. Under the identical circumstances as previously reported [24], the sample was dissolved in hexane and injected into a GC-MS (Agilent Technologies) equipped with a mass spectrometer detector (5977A) and a gas chromatograph (7890B).

3. Results and discussion

3.1. Phenotypic and genotypic characterization

S. rochei typical findings on selected media include chalky, slow growing, and different-colored aerial and substrate mycelia (Table S1). Actinomycetes differed in their physiological and biochemical properties according to the physical circumstances and the nutrients present in the medium (Table S1). The isolate showed positive results in nitrate reduction, urease decompositionand catalase activity and showed negative results in H_2S production tests. As carbon sources, it used d-glucose, sucrose, cellulose, lactose, and fructose, demonstrating its capacity to grow on a range of sugars and produce gas. While it showed weak utilization of L-tryptophane, L-tyrosine and L-asparagine.*Streptomyces*SUN35 can hydrolysis starch and casein in media. The strain SUN35 is most likely *Streptomyces* species, according to a comparison of its physiological, biochemical, and microscopic features with those of recognized actinomycetes species listed in Bergey's Manual of Systematic Bacteriology [25].

The most potent *S. rochei* that exhibited strong antimicrobial and anticancer activities was identified by molecular phylogenetic analysis. The actinomycete isolate shared 98.17% genetic similarity with *S. rochei*, according to the 16S rRNA gene sequence. The nucleotide sequence was submitted into the GenBank database under the accession number OR492299 for *S. rochei* SUN35. The evolutionary relationship between the most closely related species and the representative experimental strain can be observed in Figure (3).

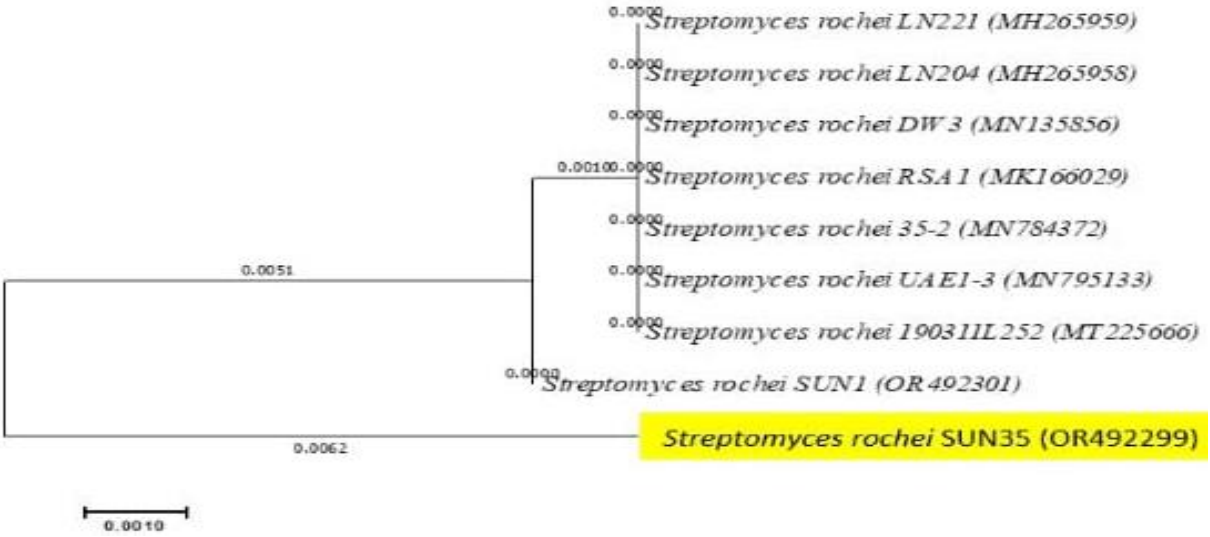

Figure 3. Evolutionary relationships of 16S rRNA sequence of *S. rochei* OR492299SUN35 with related sequences in GenBank database. The Neighbor-Joining approach was used to infer the evolutionary history. The

ideal tree is displayed with the branch length sum equal to 0.01231743. With branch lengths (next to the branches) measured in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is rendered to scale.

3.2. Screening antimicrobial activities

The antibacterial properties of the *S. rochei*EtOAc extract were assessed and compared to an antibiotic that was available (Table 1). The EtOAc extract demonstrated a 16 mm growth inhibition zone against *Staphylococcus aureus*, MRSA, and *Aspergillus fumigatus*, and a 22 mm and 23 mm inhibition zone against *Fusarium solani* and *Penicillium chrysogenum*, respectively. This percentage was expected since it has been previously documented that a significant number of actinomycetes from the soil niche exhibit antibacterial activity [26].

The EtOAc extract of *Streptomyces* sp. NMF6 exhibited moderate to strong antibacterial activity against *Vibrio damsela*, *Enterococcus faecalis*, and *Staphylococcus aureus* [27]. Additionally, in comparison to the selected isolate *S. rochei*, the EtOAc extract of *S.rochei* strain AB12 demonstrated significant antibacterial activity against *Staphylococcus aureus* and moderate to poor activity against *Pseudomonas aeruginosa* and *Aspergillus niger* [28].

Table 1. Antimicrobial activities of *S. rochei*EtOAc extract (mm).

Agent	**Zone of inhibition(mm)**					
	Antibacterial activity			**Antifungal activity**		
	Gram +ve bacteria		**Gram -ve bacteria**			
	Staphylococcus aureus	**MRSA**	***Pseudomonas aeruginosa***	***Aspergillus fumigatus***	***Fusarium solani***	***Penicillium chrysogenum***
S. rochei (100 µg/µl)	16±1.23	16±1.23	17±1.52	16±1.23	22±2.33	23±2.4
Amoxicillin/ Clavulanic acid (30 µg)	18±1.8	Negative	16±1.23	-	-	-

3.3. Screening cytotoxicity

Pharmacological research on anticancer substances from biological extracts is still very important. Thus, ongoing research has been done to find more potent natural resource anticancer extracts that might be used to create novel therapeutic medications [29]. An MTT assay was used to measure the cytotoxic activity against colon Caco-2 and liver HepG2. The mitochondrial reductase enzyme, which is frequently used to screen for cytotoxic effects, is the basis of this assay. In this investigation, Different concentrations of the crude extract *S. rochei* (200, 400, 600, 800, 1000 µg/ml) were revealed a significant concentration dependent decrease in the cell viability of colon Caco-2and liver HepG2, the cytotoxic activity lowered IC50 values to 572.5 µg/ml and 818.1 µg/ml, respectively.

The EtOAc extract effectiveness against cell lines varied, according to the results (Fig. 1). Furthermore, Metabolic extract of *S. Rochei* showed higher cytotoxicity against colon Ca**c**o-2cell line compared to liver HepG2 (Fig. 2). *Streptomyces* sp. NMF6's EtOAc extract, under accession number MW015111, demonstrated a respectable level of anticancer activity against colon HCT116 and liver

HepG2 cell lines; at 500 µg/mL, the cytotoxic activity reduced IC50 values to 49.85 and 59.39 µg/ml, respectively [30]. Additionally, the efficacy of the *Streptomyces* sp. S2A EtOAc extract (accession number KU921225) against cell lines varied. The study revealed that at a dose of 100 µg/ml, the cytotoxic activity against HT-29, MDA and U-87, and with cell inhibition was 52.31, 55.23 and 59.63 respectively [30].

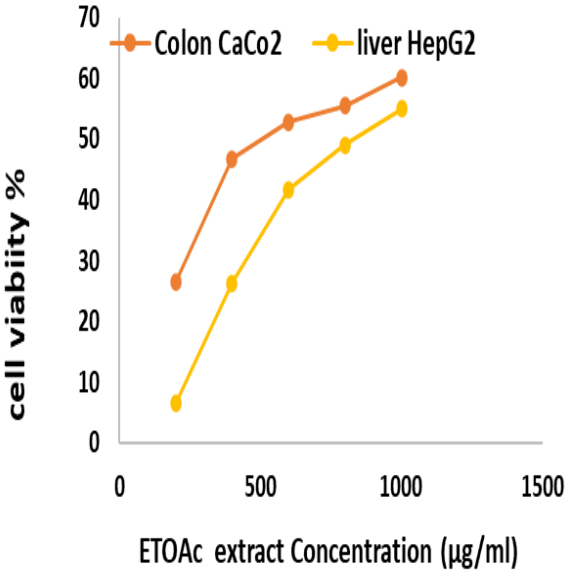

Figure 1. Cytotoxic activity of *S. rochei*EtOAc extract against colon Caco-2 and liver HepG2.

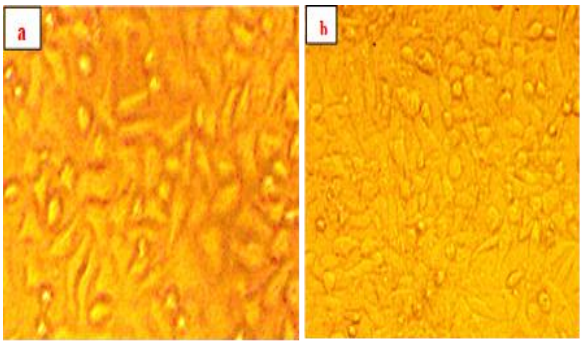

Figure 2.a. Colon Caco-2 cell line without treatment, b. Colon Caco-2 cell line after treatment with 1000 µg/ml EtOAc extract of *S. rochei*.

3.4. Gas Chromatography-Mass Spectrometry (GC-MS)

Using the GC-MS (Figure 4) as a main fingerprint, the hexane soluble components of the EtOAc extract of *S. rochei* OR492299 SUN35 were identified.

Nineteen molecules, accounting for all the mass, were identified based on the GC-MS data.

All the identified components were along with their retention times and peak area% (relative concentration) were inserted in Table 2. From all the identified constituents, 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester was characterized as the abundant compound with a relative concentration of 73.43%, followed by the two nitrogenous long chains, 13-docosenamide, (*Z*)- and 9-octadecenamide, (*Z*)- with respective relative concentrations of 5.61 and 3.13%.

The other compounds were identified as minors and/or traces. The two nonoxygenated hydrocarbons, *n*-nonadecane (0.14%) and *n*-hexadecane (0.11%), were identified with the lowest concentrations among all compounds.

The results also exhibited that this extract was rich by oxygenated compounds as well as phenolics.

Our findings were in complete harmony with the previous reports.

Several reports described that the biological active extracts from *Streptomyces pactum* [31] and *Streptomycessp*. nkm1 [32] are very rich with the phenolics and aromatic carboxylic compounds in addition to the nitrogenous and non-nitrogenous long chain compounds.

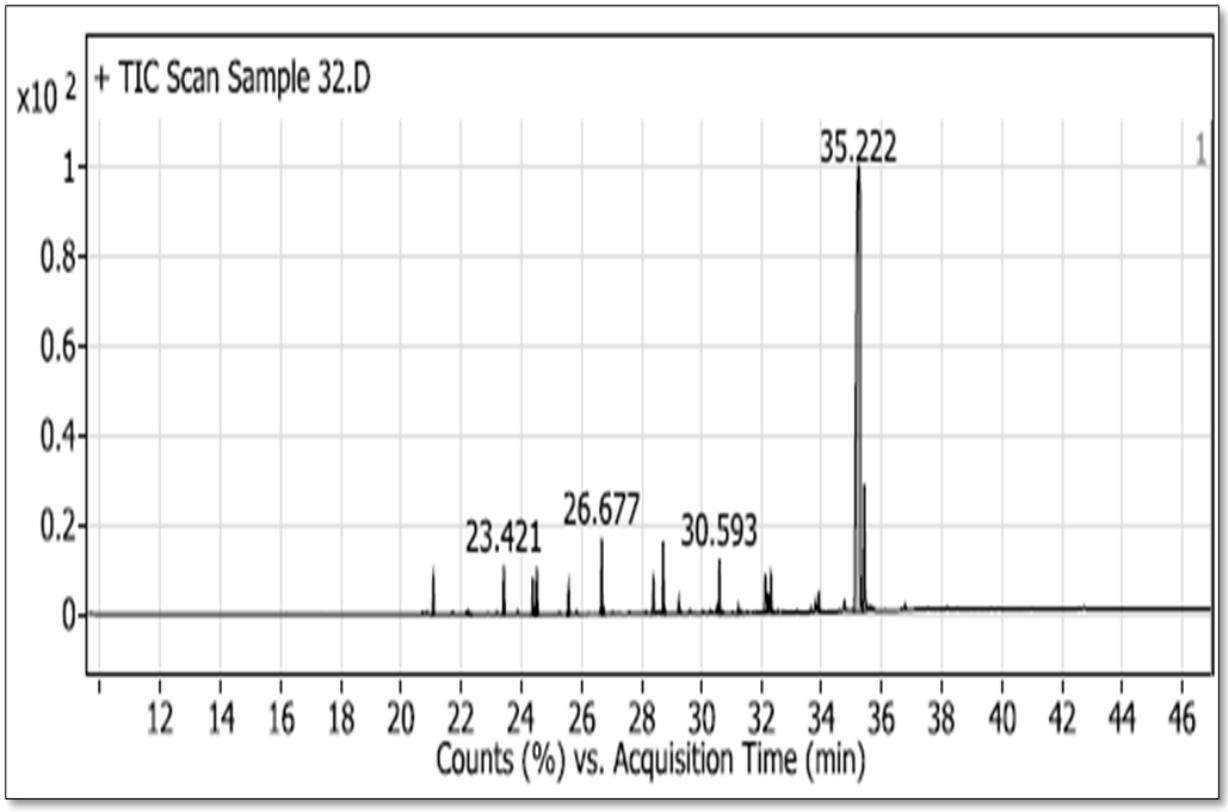

Figure 4: Ion chromatogram in GC-MS of the hexane soluble components of the EtOAc extract of *S. rochei*.

Table 2: The hexane soluble identified components of the EtOAc extract of *S. rochei*.

Peak	RT	Name	Formula	Area	Area Sum %
1	21.077	Glycerol 1,2-diacetate	$C_7H_{12}O_5$	8235532.7	1.21
2	22.238	2-Propenoic acid, 3-phenyl-	$C_9H_8O_2$	1584342.6	0.23
3	23.421	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	11369180	1.67
4	24.385	1-Hexadecanol	$C_{16}H_{34}O$	7808208.7	1.14
5	24.469	*n*-Hexadecane	$C_{16}H_{34}$	752615.57	0.11
6	24.522	Diethyl Phthalate	$C_{12}H_{14}O_4$	11337637	1.66
7	25.577	Dodecyl acrylate	$C_{15}H_{28}O_2$	6193608.8	0.91
8	26.677	1-Octadecene	$C_{18}H_{36}$	16935607	2.46
9	26.745	*n*-Octadecane	$C_{18}H_{38}$	1271892.9	0.19
10	28.4	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	9620737	1.41
11	28.726	9-Eicosene, (*E*)-	$C_{20}H_{40}$	16218424	2.38
12	28.779	*n*-Nonadecane	$C_{19}H_{40}$	981823.78	0.14
13	30.593	Behenic alcohol	$C_{22}H_{46}O$	12941772	1.90
14	31.23	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	1547008.3	0.23
15	32.126	9-Octadecenamide, (*Z*)-	$C_{18}H_{35}NO$	21370858	3.13
16	32.308	1-Tetracosanol	$C_{24}H_{50}O$	10006090	1.47
17	33.901	Hexacosyl acetate	$C_{28}H_{56}O_2$	4935289.4	0.72
18	35.222	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	501340000	73.43
19	35.419	13-Docosenamide, (*Z*)-	$C_{22}H_{43}NO$	38283928	5.61

4. Conclusion

Following its isolation from agricultural soil, *Streptomyces rochei* OR492299 has been identified through genotypic and phenotypic characterization. The EtOAc extract of the *S. rochei* was then prepared and applied to the GC-MS chemical analysis that afforded identification of 19 compounds. The oxygenated compounds represented the major components with the abundant of 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (73.43%). The S. rocheiEtOAc extract's antibacterial applications demonstrated its potent inhibitory effects on both Gram-positive and Gram-negative bacteria. Furthermore, it was found that this extract possessed potent inhibitory capabilities against the proliferation of HepG2 carcinoma cell lines and colon Caco-2 carcinoma cell lines. These findings underscore the necessity for additional pharmacological and chemical studies on this strain in the future.

Conflicts of interest
There are no conflicts to declare.

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