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STRUCTURE ELUCIDATION OF BIOACTIVE MATERIAL FROM *STREPTOMYCES SPORORAVEUS* AND ITS BIOLOGICAL ACTIVITY (PATHOGENIC MICROORGANISMS AND CYTOTOXICITY)

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ABSTRACT

Filamentous bacteria belonging to the *Streptomyces* genus are well known for their exceptional capacity to generate a diverse range of bioavailable secondary metabolites. In this work, an inoculum for fermentation was created using *Streptomyces* sp., which was isolated from Yanbu City, Saudi Arabia. It was 99% identical to *Streptomyces sporoaveus* AD 4, according to 16S ribosomal RNA sequence homology studies. After using ethyl acetate to extract the fermented broth, the crude extract's purity was checked using high-performance liquid chromatography (HPLC). Following the purification stages, three pure bioactive fractions were isolated and given the names A, B, and C, with retention times of 2.251 ± 0.3 min, 4.342 ± 0.3 min, and 5.988 ± 0.3 min, respectively. Additionally, the spectroscopic examinations of these three compounds, including their GC-MS data, were used to determine their chemical structures by comparing them to published reference data. Additionally, the three fractions exhibit potent antimicrobial activity against *Salmonella typhi* ATCC 14028, *Candida albicanc, Aspergillus niger, Klebsiella pneumoniae, Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus epidermidis, Micrococcus luteus, E. coli* ATCC 8739, and *B. subtilis* ATCC 6633. Also, the cytotoxicity of the bioactive fractions was assessed using the Hct-116 line. Of the three fractions, fraction C had the lowest cytotoxicity, with an IC50 value of 224.57 ± 5.82, followed by fraction B at 238.82 ± 2.57 and fraction A at 341.28 ± 1.69.

Keywords: *Streptomyces*, Antimicrobial activities, GC-Mass, HPLC, Anti-inflammatory

INTRODUCTION

The pharmaceutical industry is in dire need of more logical methods and techniques for the screening of novel antibiotics due to the rise in the prevalence of multi-resistant pathogenic bacteria. Many antimicrobial compounds have been produced as therapeutic agents, and antibiotics are a promising supply of these materials in the pharmaceutical industry **(Berdy, 2005)**. Research on antimicrobial resistance is currently urgently needed, and new bioactive substances are required to fight these infections.

Natural products present prospects for the discovery of the most dependable and promising source of physiologically active molecules with more chemical and structural diversity is novel therapeutic lead compounds that exhibit broad-range target activity **(Harvey *et al*., 2001; Tripathi *et al*., 2004; Singh *et al*., 2012)**. The majority of secondary metabolites are produced by microorganisms, which are also an invaluable reservoir of novel secondary metabolites that host an array of biological applications **(Vining, 1990)**. About 75% of antibiotics that are used in medicine are made by various Streptomyces species, and the majority of known antibiotics are derived from the actinomycetes group of bacteria **(Gogoi *et al*., 2005; Sharma, 2014)**. The actinomycetes *Streptomyces* is well known for its remarkable ability to generate various bioactive chemicals. The scientific community has given this species of filamentous bacteria a great deal of attention because of its capacity to produce a broad variety of secondary metabolites that have practical applications in medicine.

As a result, a focus of study for a long time has been on the screening, isolation, and characterisation of actinomycetes that show promise in developing future antibiotics **(Singh *et al*., 2008)**. Gram-positive aerobic bacteria belonging to the actinomycetales order in the Actinobacteria class are called *streptomyces*. In 1943, Waksman and Henrici made the initial proposal for the genus *Streptomyces*. Its shape and cell wall chemotype led to its classification into the Streptomycetaceae family **(Waksman and Henrici, 1943)**.

Among actinomycetes, the genus *Streptomyces* is a significant group of organisms in terms of the economy and serves as the primary source of a variety of biologically active chemicals **(Berdy, 2005)**. *Streptomyces* sp. was the source of over 75% of all known antibiotics that are helpful in medicine and commerce, as well as other chemicals that are significant to agriculture **(Cundlife, 1989)**. Additionally, *Streptomyces* species are the most abundant source of new chemicals with a wide range of bioactivities among actinomycetes **Singh *et al*., 2012; Vining, 1990)**.

The primary goal of this investigation was to identify the antibacterial substances generated by the soil isolate *Streptomyces sporoaveus* DA 4. Using GC-MS and HPLC analysis, the antibacterial compounds were isolated and characterised. Conversely, cytotoxicity was also investigated.

MATERIALS AND METHODS

Materials

In the current investigation, all of the compounds were 99% pure, and the solvents were HPLC grade. Sigma Aldrich was the source of the microbiological culture medium employed.

Methods

Isolation of *Streptomyces* strains

The samples were brought in an insulated container to a laboratory for pre-treatment and subsequent usage after being collected at a depth of 1 to 10 cm from several locations in the Yanbu region of Saudi Arabia. Samples were taken and allowed to air dry for a week, then stored for two days at 50°C in a hot air oven. Samples were diluted up to a maximum of 10^{-6} serially. On starch casein agar, aliquots of each dilution (100 µl) were added, which was treated with nystatin (25 µg/mL) and nalidixic acid (20 µg/mL). For one or two weeks, at 28°C, the plates were incubated. The plates' growth was observed, and on (ISP-2) International Streptomyces Project-2 slants pure colonies were maintained and kept cold (-4°C) for later use. The medium was used to select and purify actinomycetes cultures based on colony morphology. We isolated and dubbed AD 20 a total of 20 actinomycetes from our pilot scale screening **(El-Ghwas and El-Waseif, 2016).**

Cross streak technic

Cross-streak analysis was utilised to assess the antibacterial activity of actinomycetes isolates **(Oskay, 2009)**. Seven days of incubation at 30oC followed the infection of YPG plates with isolates using a single stripe in the centre of the Petri plate. Following a single streak inoculation at a 90° angle to the actinomycetes strains, the plates were incubated for an entire night at 37°C with the test organisms. The test organism's inhibition revealed antagonistic relationships. The results are shown as follows: (M) moderate activity (50%), (W) weak activity (25%), (G) good activity (100% activity); and - no activity.

Genetic identification for the most potent strain

50 millilitres of ISP2 medium without agar were infected with the most potent strain of *Streptomyces* sp. that exhibited strong antibacterial activity. The medium was then continuously shaken for 5-7 days, or until the turbidity reached a high level. After centrifuging the cells at 10000 g for 20 minutes at 4°C, they were congealed (no supernatant) in 2 mL Eppendorf tubes at -20°C until they were needed **(Hei *et al*., 2021; Al-Janabi *et al*., 2022a)**. DNA extraction was carried out following manufacturer instructions using automated equipment (Mag Purix Bacterial DNA Extraction Kit). The NanoDrop 8000 Spectrophotometer from Thermo Scientific was used to quantify the extracted DNA from the isolate, which was then stored at -20°C until needed. Adjusted for the PCR process, the DNA sample was 100 ng/µL.

Using the universal bacterial primers Fd1 (5′-AGA GTT TGA TCA TGG CTC AG-3′) and rP2 (5′-ACG GTT ACC TTG TTA CGA CTT-3′), the 16S rRNA gene was amplified to produce an amplicon of 1500 bp under the specified circumstances **(Weisburg *et al*., 1991)**. The amplified fragment underwent electrophoresis analysis. To be more exact, 8 µL of PCR products were placed with the 1 kb molecular weight marker (thermoscientific) on a 1% agarose gel.

Exo SAP-IT PCR cleanup kit (Applied Biosystems) was used to purify the amplified product, and Cycle Sequencing Kit for the Big Dye Terminator (Applied Biosystems) version 3.1 was utilised for the sequencing reaction. Sequencing products were the parameters specified by the manufacturer and transferred onto an ABI 3130xL capillary sequencer (Applied Biosystems). After being through the utilisation of the BigDye® Xterminator TM Purification Kit (Applied Biosystems). The Sigma Company performed the extraction, amplification, and sequencing tasks.

Using the p-distance approach and 1000 bootstrap repetitions for every cluster, the NJ phylogenetic tree—which illustrates the evolutionary relatedness of the strains—was constructed by aligning the assembled sequences using MEGA 11 software **(Al-Janabi *et al*., 2022b; Qadah *et al*., 2023; Tamura *et al*., 2021)**. The EZBio Cloud database was used to identify the most closely related sequences. (https://www.ezbiocloud.net).

Fermentation, Extraction and Purification of bioactive fraction

Fermentation and Extraction

A separate inoculation of 100 ml of the isolated strain DN 4 culture broth was made in 1000 ml of the International Streptomyces Project-2 medium (ISP-2). For 21 days at 28–30°C, the infected flask was incubated in a shaker set at 200 rpm. The culture broth for the isolated stain was centrifuged at 8000 rpm for 15 minutes after the fermentation process was finished to extract the cell debris. Following the collection of the culture filtrate, the secondary metabolites were extracted using a solvent. For full extraction, a shaker was used to agitate the mixture overnight after adding ethyl acetate to the culture filtrate in a ratio of 1:1 (v/v). A rotary evaporator (Laboratory 4,000) was used to concentrate the solvent phase containing the strain's crude fraction after it had been separated from the aqueous phase. The dried active crude fraction was then gathered and kept for future study in a refrigerator.

Purification

High-performance liquid chromatography (HPLC)

HPLC is a highly well-liked technique that is frequently used to quantify and separate chemicals to identify and isolate naturally occurring bioactive molecules. With the analytical grade Hypersil BDS C-18 column (250 mm x 4.6 mm, 5µm), the crude extract and column fractions of the crude extract produced from *Streptomyces spororaveus* DA 4 were analysed by HPLC. After combining the dried extracts with methanol to create a sample solution, the solids were fully dissolved in the solution by sonicating it for 20 minutes. A combination of 0.1% acetonitrile (solvent A) and formic acid (solvent B) in a 70:30, v/v ratio was used as the mobile phase for the isocratic elution mode. 20 µL of injection volume, 8 minutes of run time, and 1 ml per minute^{-1} flow rate were used. A UV detector with a 254 nm wavelength was used to track the compound's elution.

GC-Mass spectroscopy

Using a gas chromatography GC–MS system (GC: 5890 series II; MSD 5972), The volatile component profile was evaluated in the crude ethyl acetate extract of the DA-4 strain. Mass spectrometry (MS) was directly connected to a fused-silica HP-5 capillary column (0.25 mm diameter, 30 m long, and 0.25 mm film thickness). The carrier gas was helium, which flowed at a rate of 1.2 ml/min. The oven was programmed to function at 50ºC for one minute, followed by a five-degree Celsius increase to 280ºC. per minute, and it was maintained at this temperature isothermally for 20 minutes. At 280°C for the detector and 250°C for the injector port, the temperatures were kept consistent. Mass-spectral analysis was performed on the peaks of the components obtained from gas chromatography. The components were discovered by comparing their mass spectra to those of the WILEY 09 and NIST 14 mass spectral databases **(Abd El-Kareem *et al*., 2016; Elhalabi *et al*., 2024)**.

Antimicrobial activity of fractions

As previously reported by **Siddharth and Vittal (2018)**, the antibacterial activity of the active fractions (A, B, and C) for the isolated strain DA 4 was investigated using the disc-diffusion method. *Salmonella typhi* ATCC 14028, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 6538 were the pathogenic bacteria that were tested. In addition, we examined clinical isolates of *Aspergillus niger*, *Candida albicans*, *Klebsiella pneumoniae*, *Micrococcus luteus*, and *Staphylococcus epidermidis*. The examined bacteria underwent a 72-hour incubation period at 37°C after being sub-cultured on nutrient agar. The examined fungi were then sub-cultured on a PDA medium and given a 10-day incubation period at 28°C. A spectrophotometer calibrated for a wavelength of 623 nm was used to modify the optical density of each inoculum to produce an optical density of 0.18 to 0.20, or roughly 10^6 spore/mL **(Bastide *et al*., 1986)**. As a positive control, a disc containing ethyl acetate in an amount equivalent to the extract was utilised. After incubating the bacteria at 37°C for 24 hours and the fungus at 28°C for 48 hours, the diameter of the inhibitory zones was determined.

Determination of sample cytotoxicity

To form a complete monolayer sheet, the 96-well tissue culture plate was inoculated with 1 X 10^5 cells/ml (100 ul/well), and the cytotoxicity of compounds A, B, and C was evaluated on this plate for 24 hours. Following the formation of a confluent sheet of cells, the growth material was withdrawn from the 96-well microtiter plates and the cell monolayer was washed twice with wash media. The test sample was diluted twice before being placed in an RPMI medium containing 2% serum (the maintenance medium). Each dilution was tested in 0.1 ml increments in several three wells that acted as controllers and received only maintenance media. The plate was tested after being incubated at 37°C. Physical indicators of toxicity, like rounding, shrinkage, granulation, or partial or whole loss of the monolayer, were examined in the cells. (5 mg/ml in PBS) was used to make the MTT solution (BIO BASIC CANADA INC). Each well was given 20 µl of the MTT solution. To mix the MTT into the medium, place it on a shaking table and shake for five minutes at 150 rpm. To facilitate the metabolism of MTT, subject it to a four-hour incubation period at 37°C with 5% CO_2. Remove any remaining residue by emptying the medium and, if necessary, drying the plate with paper towels. The metabolic product formazan of MTT should be dissolved in 200 µl of dimethyl sulfoxide (DMSO). To fully homogenise the formazan and solvent, place it on a shaking table and agitate it for five minutes at a speed of 150 revolutions per minute. The background at 620 nm should be subtracted, and the optical density at 560 nm should be measured. There ought to exist a direct correlation between the quantity of cells and the optical density **(El-Waseif and El-Ghwas, 2022)**.

RESULTS

In the last few years, actinomycetes have been thoroughly investigated in several studies conducted in diverse and harsh habitats, niches, and conditions across the globe. To isolate actinomycetes strains for our investigation, soil samples were gathered from various locations within the Yanbu region of Saudi Arabia. Twenty pure cultures of suspected actinomycetes were placed on ISP-2 slants based on colony shape and stability during subculturing. Since the pathogenic microbes studied (*S. aureus ATCC 6538*, *E. coli ATCC 8739*, *B. subtilis ATCC 6633*, *B. subtilis ATCC 6633*, *S. typhi ATCC 14028*, and *B. subtilis ATCC 6633*) most actinomycetes have been shown to have antibiotic action. Additionally, as indicated in Table (1), we evaluated clinical isolates of *Aspergillus niger*, *Candida albicans*, *Klebsiella pneumoniae*, *Micrococcus luteus*, and *Staphylococcus epidermidis*. Five (25%) showed weak activity, seven (35%) showed moderate activity, two (10%) showed good activity, and six (30%) showed no antagonistic activity during the initial screening. Additionally, among the strains examined, DA 4 demonstrated high antibacterial activity against all tested pathogens, as shown in **Table 1**. Therefore, 16s rDNA amplification and sequencing were used to identify this strain.

Table 1 The initial evaluation of actinomycetes isolates was conducted utilising the cross-streak technique.

	Gram (+ve) Bacteria				Gram (-ve) Bacteria				Fungi	
Isolate	***S. epidermis Clinical isolate***	***S. aureus ATCC 6538***	***B. subtilis ATCC 6633***	***M. Luteus Clinical isolate***	***E. coli ATCC 8739***	***K. pneumoniae Clinical isolate***	***P. aeruginosa ATCC 9027***	***S. typhi ATCC 14028***	***C. albicanc Clinical isolate***	***A. niger Clinical isolate***
DA 1	W	W	W	-	W	-	W	W	W	-
DA 2	-	-	-	-	-	-	-	-	-	-
DA 3	M	M	W	W	W	W	W	M	M	M
DA 4	G	G	G	G	-	G	G	G	G	G
DA 5	-	-	-	-	-	-	-	-	-	-
DA 6	-	-		W	W	W	-	W	-	W
DA 7	W	G	-	M	W	-	W	W	W	W
DA 8	-	-	-	-	-	-	-	-	-	-
DA 9	M	M	G	-	M	-	M	-	M	G
DA 10	-	G	-	G	M	M	M	-	M	M
DA 11	W	-	M	-	W	W	W	-	W	-
DA 12	G	M	G	-	G	M	W	G	W	G
DA 13	-	M	_	W	M	_	W	M	-	M
DA 14	M	G	W	-	W	-	-	M	M	M
DA 15	-	-	-	-	-	-	-	-	-	-
DA 16	W	M	-	-	M	M	-	M	-	G
DA 17	G	M	-	-	M	-	-	-	M	M
DA 18	-	-	-	-	-	_	-	-	-	-
DA 19	-	W	M	-	-	-	-	W	W	W
DA 20	-	-	-	-	-	-	-	-	-	-

**(M) moderate activity (50%), (W) weak activity (25%), (G) good activity (100% activity); and - no activity.*

Molecular characterization of DA 4 strains

According to the results of Blast analysis and 16S rDNA sequencing, isolate DA 4 was identified as belonging to the *Streptomyces* species (bases 1e1470 linear DNA). **Figure 1** shows that the strain and *Streptomyces spororaveus's* 16S rRNA genes had 99% similarity.

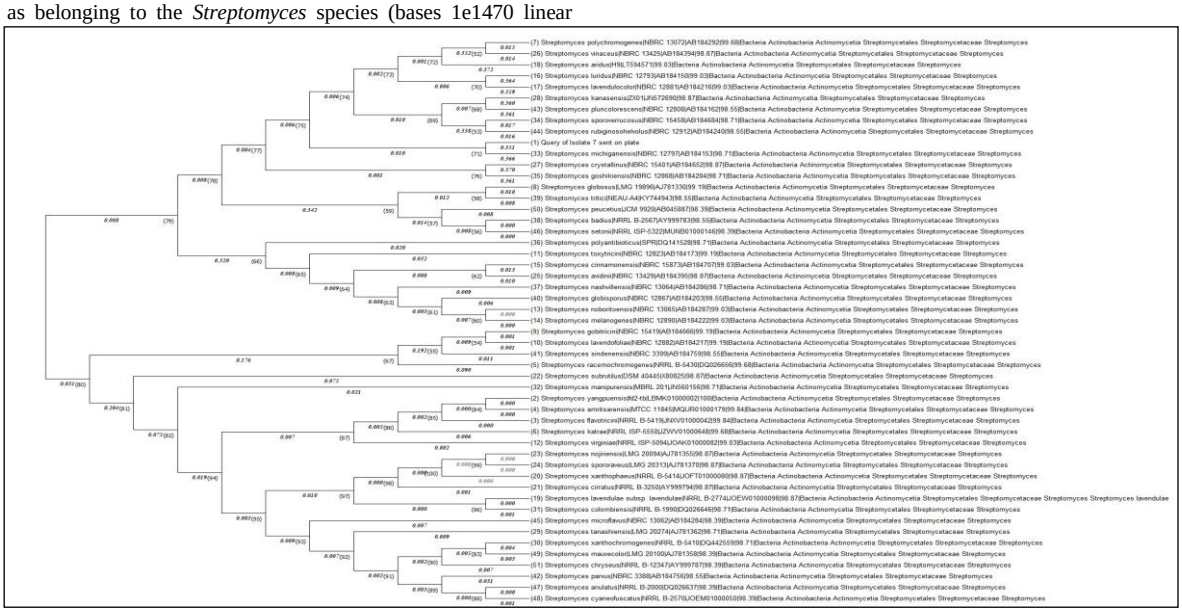


Figure 1 Phylogenetic tree illustrating the evolutionary distances and relationships between *Streptomyces spororaveus* DA 4 and other *Streptomyces* species.

Fermentation and extraction

As indicated in the material and procedure sections, the fermentation process was run for seven days at 30°C with shaking conditions utilising the ISP-2 medium. To remove the biomass from the broth, the fermented culture was filtered using a Buchner funnel. Thus, two extractions of the cell-free broth were made using ethyl acetate at a ratio of 1:1 (v/v). A rotary evaporator from Buchi Laboratories in Switzerland concentrated the organic phase until it was completely dry under a vacuum.

Purification of active compounds

Determining the amount of crude extract powder is measured by HPLC by weighing 500 mg of crude extract in 100 ml of methanol and allowing it to dissolve completely before measuring the amount of crude extract. The extract was revealed to contain a combination of three active chemicals, designated A, B, and C, after purification and HPLC measurement. A peak was seen at 2.251 ± 0.3 min for fraction A (190 mg/gm), followed by fraction B (140 mg/gm) at 4.342 ± 0.3 min, and fraction C (170 mg/gm) at 5.988 ± 0.3 min. **Figure 2** displays the acquired chromatograms of the three fractions.

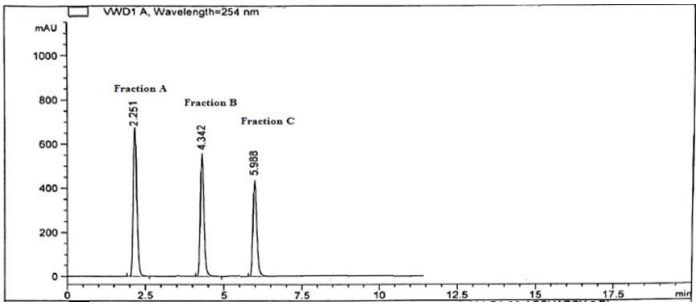


Figure 2 The pure chemicals A, B, and C are seen in the HPLC profile to have eluted at 2.251, 4.342, and 5.985 minutes, respectively.

Conversely, the three fractions' physicochemical characteristics were as follows: Fraction A was separated and found to be a white powder that was soluble in methanol, ethanol, and dimethyl form amide. It was discovered that Fraction A melting point was 182.5ºC. On the other hand, Fraction B was identified as a white to beige powder that had a melting point of 256.8ºC and was soluble in methanol, DMSO, and acetonitrile. The fraction C white crystalline powder was finally achieved, and it was discovered to have a melting point of 200ºC and to be soluble in DMSO, dimethyl form amide, and methanol. HPLC was used to demonstrate the purity of fractions A, B, and C, which were determined to be respectively 97.5%, 98%, and 96.5%.

GC-Mass analysis

On the other hand, GC/MS purification, which is widely regarded as one of the most accurate analyses available, was applied to the three fractions A, B, and C. Analysis of *Streptomyces spororaveus* DA 4 (**Figures 3-5, Table 2-4**) revealed the presence of three fraction A, B, and C. It is especially useful. This material is suitable for applications that need low detection limits and can be employed to assess samples in various chemical states, regardless of their size, even when the quantity of the sample is restricted.

Twelve compounds were found in fraction A in **Figure 3** and **Table 2**, making up 97.14% of the extract composition overall. These compounds are as follows: hexadecam ethylcyclooctasiloxane (0.33%), Glycine N-[(3à,5á,7à,12à)-24-oxo-3,7,12tris[(trimethylsilyl)oxy] cholan-24-yl]-, methyl ester (0.03%), Tetradecamethylcycloheptasiloxane (0.08%), 1h-phenalen-1-one-9-hydroxy (5.58%), 11Oxatricyclo [5.3.0.1(2,6)] undecane-4-one, 3-endo-5-endo-dimethyl-9 iso propylidene (0.16%), Benzazepin-1-one, 1, 2, 3, 4-tetrahydro-N-acetyl-7,8,9-trimethoxy (46.90%), 10-Octadecenoic acid, methyl ester (0.31%), Hexadecanoic acid, 2, 3-dihydroxy propyl ester (23.51%), 2-hydroxy-3 -[(9E)-9-octadecenoyloxy] propyl (9E)-9- octadecenoate (1.51%), 9-octadecenoic acid (Z)-,2[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy]methyl] ethyl ester (0.08%), Ethyl 3,7,12-trihydroxycholan-24-oate (8.25%), and 7,8-Epoxylanostan-11-ol, 3-acetoxy (10.40%). Fraction B in **Figure 4** and **Table 3** revealed the existence of eighteen chemicals, which make up 95.32% of the overall composition of the extract as follow: Hexadecanoic acid, methyl ester (7.82%), n-Hexadecanoic acid (30.56%), Octadecanoic acid, methyl ester (5.21%), Octadecanoic acid (1.93%), Glycidyl palmitate (0.89%), Glycidol stearate (8.79%), Octadecanamide (0.73%), Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (0.75%), 2,3Bis [(trimethylsilyl) oxy] propyl Palmitate (5.83%), N-((E)-(3S)-Benzylamino-5-phenyl-4 penenoyl)bornane-10,2-saltam (5.59%), 2-Monolinoleoylglycerol trimethylsilyl ether (0.64%), 2,3Bis [(trimethylsilyl) oxy] propyl stearate (6.41%), (Z)-9-octadecenamide (Adogen 73), (1.03%), Dimethyl(bis[(4,8,8-trimethyldecahydro-1,4-methanoazulen-9-yl)methoxy])silane (0.58%), Rhodamine 6G cation (5.03%), 2-(Octadecyloxy) ethyl sterate (1.38%), Methyl 3-oxooctadecanoate (3.71%) and 13-Docosenamide, (Z)- Erucylamide (8.44%). Fraction C in **Figure 5** and **Table 4** was illustrated that there were twenty-two compounds present, which accounted for 94.41% of the total content of the extract as follow: 2-Pentanone, 4-hydroxy-4-methyl Acetonyl dimethyl carbinol (0.19%), Dodecamethylcyclohexasiloxane (0.07%), Cycloheptasiloxane, tetradecamethyl (0.15%), 10,13-Octadecadiynoic acid, methyl ester (0.08%), Decane, 1-phenyl (0.1%), Linoleoyl chloride 9,12-Octadecadienoyl chloride, (Z,Z) (0.07%), Oleic Acid 9-Octadecenoic acid (Z) (0.08%), 1-hexadecanol, 2-methyl (0.08%), 1-Eicosanol (0.07%), Cyclooctasiloxane, hexadecamethyl (0.07%), Tridecanedial (0.06%), Palmitaldehyde (0.1%), 7-Octadecenoic acid, methyl ester (0.53%), Glycidyl palmitate (5.28%), L-(+)-ascorbic acid 2,6-dihexadecanoate (2.1%), 1-cis-Vaccenoylglycerol (1.2%), Butyl 9,12-octadecadienoate (8.75%), Cycloheptasiloxane tetradecamethyl (4.8%), Trimethoprim (65.56%), 12-Hydroxyoleanan-3-yl acetate (3.86%), 9,12-Octadecadienoic acid (Z, Z)-, 1,2,3-propanetriyl ester, (Trilinolein) (0.28%), and Oleic acid, 3-(octadecyloxy) propyl ester (1.21%).

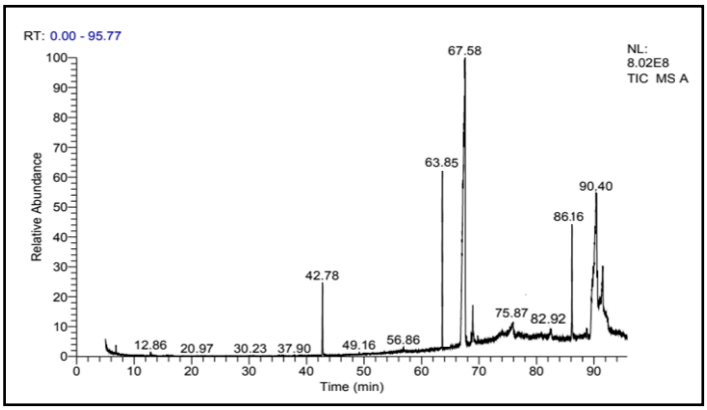
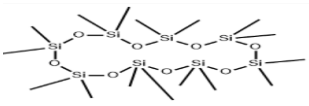
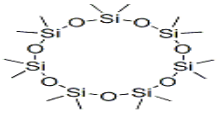
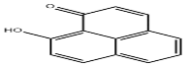
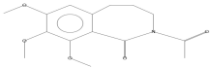
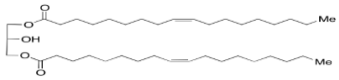
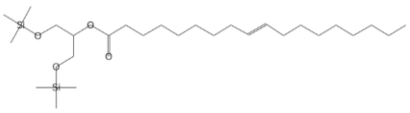
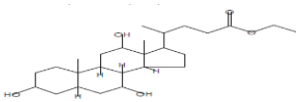
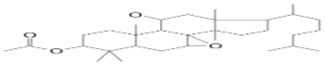


Figure 3 GC-Mass of fraction (A) obtained from *Streptomyces Spororaveus* DA 4 ethyl acetate extract

Table 2 The primary bioactive constituents detected in fraction A of the *Streptomyces Spororaveus* DA 4 ethyl acetate extract were identified via GC-MS analysis.

Retention Time (RT)	Compound name	Structure	Chemical formula	Molecular weight (MW)	Peak Area (%)
12.86	hexadecamethylcyclooctasiloxane		$C_{16}H_{48}O_8Si_8$	592	0.33
20.97	Glycine, N-[(3à,5á,7à,12à)-24-oxo-3,7,12tris[(trimethylsilyl)oxy] cholan-24-yl]-, methyl ester		$C_{36}H_{69}NO_6Si_3$	695	0.03
37.90	Tetradecamethylcycloheptasiloxane		$C_{14}H_{42}O_7Si_7$	518	0.08
42.78	1h-phenalen-1-one-9-hydroxy		$C_{13}H_8O_2$	196	5.58
49.16	11Oxatricyclo [5.3.0.1(2,6)] undecan-4-one, 3-endo-5-endo-dimethyl-9-isopropylidene		$C_{15}H_{22}O_2$	234	0.16

Continue Table 2

67.58	10-Octadecenoic acid, methyl ester		$C_{19}H_{36}O_2$	296	46.90
56.86	Benzazepin-1-one, 1,2,3,4-tetrahydro-N-acetyl-7,8,9-trimethoxy		$C_{15}H_{19}NO_5$	293	0.31
63.85	Hexadecanoic acid, 2, 3-dihydroxy propyl ester		$C_{19}H_{38}O_4$	330	23.51
75.87	2-hydroxy-3 -[(9E)-9-octadecenoyloxy] propyl (9E)-9- octadecenoate		$C_{39}H_{72}O_5$	620	1.51
82.92	9-octadecenoic acid (Z)-, 2[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy] methyl] ethyl ester		$C_{27}H_{56}O_4Si_2$	500	0.08
86.16	Ethyl 3,7,12-trihydroxycholan-24-oate		$C_{26}H_{44}O_5$	436	8.25
90.40	7,8-Epoxylanostan-11-ol, 3-acetoxy		$C_{32}H_{54}O_4$	502	10.40
Total area%					97.14

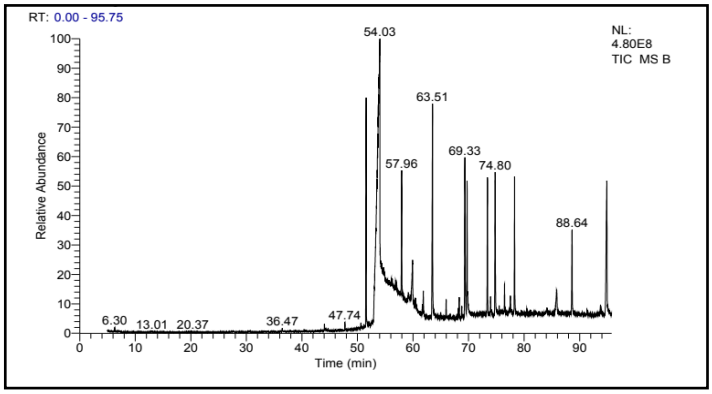


Figure 4 GC-Mass of fraction B from *Streptomyces Spororaveus* DA 4 ethyl acetate extract

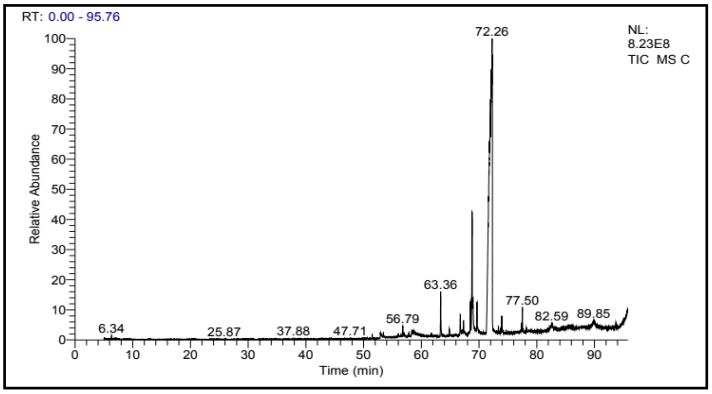
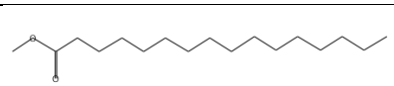
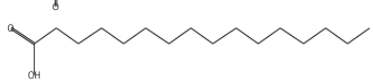
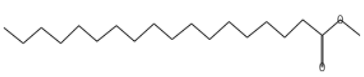
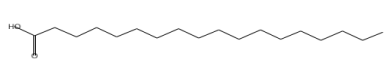
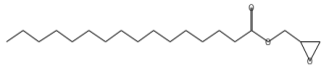
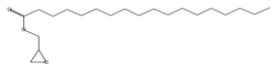
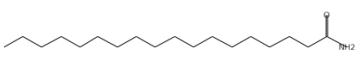
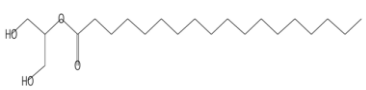


Figure 5 GC-Mass of *Streptomyces Spororaveus* DA 4 fraction C derived from ethyl acetate extract

Table 3 The primary bioactive constituents detected in fraction B of the *Streptomyces Spororaveus* DA 4 ethyl acetate extract were identified via GC-MS analysis.

Retention Time (RT)	Compound name	Structure	Chemical formula	Molecular weight (MW)	Peak Area (%)
51.56	Hexadecanoic acid, methyl ester		$C_{17}H_{34}O_2$	270	7.82
54.03	n-Hexadecanoic acid		$C_{16}H_{32}O_2$	256	30.56
57.96	Octadecanoic acid, methyl ester		$C_{19}H_{38}O_2$	298	5.21
59.92	Octadecanoic acid		$C_{18}H_{36}O_2$	284	1.93
61.85	Glycidyl palmitate		$C_{19}H_{36}O_3$	312	0.89
63.51	Glycidol stearate		$C_{21}H_{40}O_3$	340	8.79
65.97	Octadecanamide		$C_{18}H_{37}NO$	283	0.73
68.32	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester		$C_{21}H_{42}O_4$	358	0.75

Continue Table 3

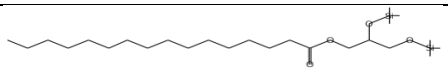
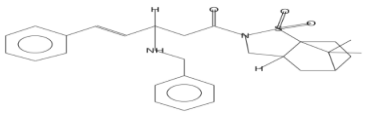
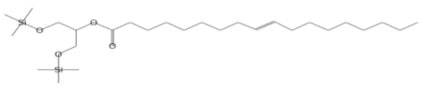
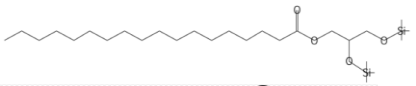
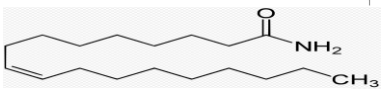
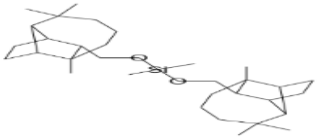
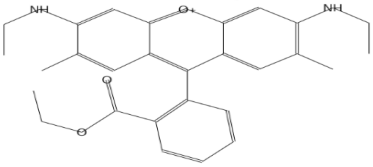
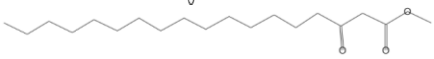
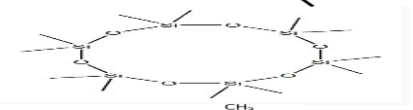
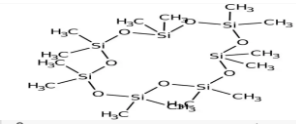
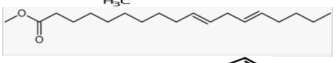
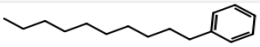
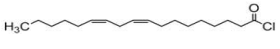
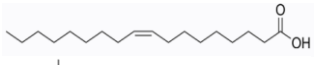
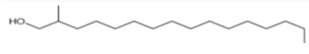
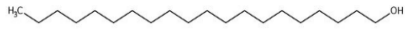
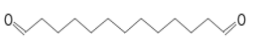
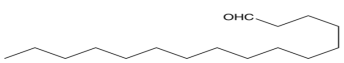
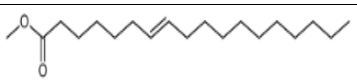
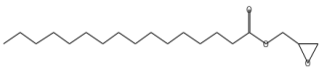
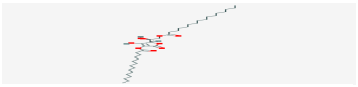
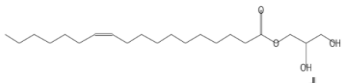
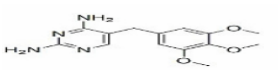
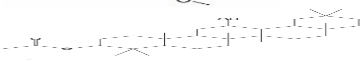
69.33	2,3Bis [(trimethylsilyl) oxy] propyl Palmitate		$C_{25}H_{54}O_4Si_2$	474	5.83
73.40	N-((E)-(3S)-Benzylamino-5-phenyl-4 penenoyl) bornane-10,2-saltam		$C_{28}H_{34}N_2O_3S$	478	5.59
73.97	2-Monolinoleoylglycerol trimethylsilyl ether		$C_{27}H_{54}O_4Si_2$	500	0.64
74.79	2,3Bis [(trimethylsilyl) oxy] propyl stearate		$C_{27}H5_8O_4Si$	502	6.41
76.48	(Z)-9-octadecenamide (Adogen 73)		$C_{18}H_{35}NO$	281	1.03
77.53	Dimethyl(bis[(4,8,8-trimethyldecahydro-1,4-methanoazulen-9-yl) methoxy]) silane		$C_{32}H_{56}O_2Si$	500	0.58
78.28	Rhodamine 6G cation		$C_{28}H_{31}N_2O_3$	443	5.03
85.86	2-(Octadecyloxy) ethyl sterate		$C_{38}H_{76}O_3$	580	1.38
88.63	Methyl 3-oxooctadecanoate		$C_{19}H_{36}O_3$	312	3.71
94.89	13-Docosenamide, (Z)-Erucylamide		$C_{22}H_{43}NO$	337.5	8.44
Total area%					95.32

Table 4 The primary bioactive constituents detected in fraction C of the *Streptomyces Spororaveus* DA 4 ethyl acetate extract were identified via GC-MS analysis.

Retention Time (RT)	Name of compound	Structure	Chemical formula	Molecular weight (MW)	Peak Area (%)
6.34	2-Pentanone, 4-hydroxy-4-methyl Acetylmethylcarbinol		$C_6H_{12}O_2$	116	0.19
25.87	Dodecamethylcyclohexasiloxane		$C_{12}H_{36}O_6Si_6$	444	0.07
37.87	Cycloheptasiloxane, tetradecamethyl		$C_{14}H_{42}O_7Si_7$	518	0.15
38.90	10,13-Octadecadiynoic acid, methyl ester		$C_{19}H_{30}O_2$	290	0.08
42.79	Decane, 1-phenyl		$C_{16}H_{26}$	218	0.10
43.06	Linoleoyl chloride 9,12-Octadecadienoyl chloride, (Z,Z)		$C_{18}H_{31}ClO$	298	0.07
43.43	Oleic Acid 9-Octadecenoic acid (Z)		$C_{18}H_{34}O_2$	282	0.08
43.65	1-hexadecanol, 2-methyl		$C_{17}H_{36}O$	256	0.08
44.03	1-Eicosanol		$C_{20}H_{42}O$	298	0.07
44..31	Cyclooctasiloxane, hexadecamethyl		$C_{16}H_{48}O_8Si_8$	592	0.07
46.78	Tridecanedial		$C_{13}H_{24}O_2$	212	0.06
47.71	Palmitaldehyde		$C_{16}H_{32}O$	240	0.10

Continue Table 4

56.79	7-Octadecenoic acid, methyl ester		$C_{19}H_{36}O_2$	296	0.53
63.36	Glycidyl palmitate		$C_{19}H_{36}O_3$	312	5.28
66.75	L-(+)-ascorbic acid 2,6-dihexadecanoate		$C_{38}H_{68}O_8$	652.9	2.1
67.31	1-cis-Vaccenoylglycerol		$C_{21}H_{40}O_4$	356	1.2
68.48	Butyl 9,12-octadecadienoate		$C_{22}H_{40}O_2$	336	8.75
69.64	Cycloheptasiloxane, tetradecamethyl				4.8
72.26	Trimethoprim		$C_{14}H_{18}N_4O_3$	290	65.56
77.50	12-Hydroxyoleanan-3-yl acetate		$C_{32}H_{54}O_3$	486	3.86
82.59	9,12-Octadecadienoic acid (Z,Z)-, 1,2,3-propanetriyl ester (Trilinolein)		$C_{57}H_{98}O_6$	878	0.28
89.85	Oleic acid, 3-(octadecyloxy)propyl ester		C39H76O3	592	1.21
Total area%					94.41

Antimicrobial activity

The antibacterial activity of each of the three fractions was examined about the test organisms that were the subject of the investigation. At a 2.5 µg/disc concentration, the antibacterial activity of the three fractions of *Streptomyces spororaveus* DA 4 was evaluated against 4 Gram (+ve) and 4 Gram (-ve) bacteria as well as 2 harmful fungi, as shown in **Table 5**. Except for *Escherichia coli* ATCC 8739, all three fractions had broad spectrum activity as seen by a distinct zone of inhibition on Muller-Hinton agar plates (MHA). Furthermore, the three fractions affect the bacteria under investigation that are Gram-positive more than Gram-negative. Fraction A demonstrated the greatest antimicrobial activity against the Gram (+ve) bacteria *Staphylococcus aureus* ATCC 6538, with an inhibition zone of 50.0 mm. *Staphylococcus epidermidis* came in second with an inhibition zone of 48 mm, followed by *Micrococcus Luteus* with a 45.0 mm inhibition zone and *Bacillus subtilis* with a 40.7 mm inhibition zone. While *Salmonella typhi* and *Klebsiella pneumoniae* had good activity with inhibition zones of 20.0 and 11.5 mm, respectively. Gram-negative bacteria did not yield any results when tested on *Escherichia coli* and *Pseudomonas aeruginosa* using fraction A. On the other hand, the inhibitory zone diameter of fraction A was found to be 28.0 and 11.0 nm for *Candida albicans* and *Aspergillus niger*, respectively. Furthermore, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* were all shown to be somewhat resistant to the effects of faction B on Gram-positive bacteria, with respective inhibition zones of 32.0, 30.4, 25.0, and 20.3 mm. However, fraction B demonstrated moderate antibacterial efficacy of 20.3 and 15 mm on *Pseudomonas aeruginosa* and *Salmonella typhi* ATCC, but no effect on *Escherichia coli*. Furthermore, *Candida albicans* exhibits modest antimicrobial activity of 15.0 mm, while *Aspergillus niger* displays good antimicrobial activity of 25.0 mm. Additionally, fraction C exhibited the strongest antimicrobial activity against the Gram-positive Bacillus subtilis, with an inhibition zone of 40.8 mm. This was followed by *Micrococcus Luteus*, which had an inhibition zone of 34.0 mm, and *Staphylococcus aureus* and *Staphylococcus epidermidis*, which had inhibition zones of 30.7 and 25.0 mm, respectively. However, the following illustrates how percent C affects Gram (-ve) bacteria: *Klebsiella pneumoniae*, *Salmonella typhi*, and *Pseudomonas aeruginosa* have inhibition zones of 20.5, 20.3, and 20.0 mm, respectively. However, fraction C displayed no *Escherichia coli* activity. Conversely, fraction C demonstrated antibacterial efficacy against *Aspergillus niger*, exhibiting an inhibition zone of 28.0 mm, and *Candida albicans*, exhibiting an inhibition zone of 13.0 mm, respectively.

Table 5 The antimicrobial activity of three fractions from *Streptomyces spororaveus* DA 4 against pathogenic microorganisms under study

Microorganisms		**Inhibition zone of Fractions (mm)**		
		A	**B**	**C**
Gram-Positive Bacteria	*Staphylococcus aureus* ATCC 6538	50.0 ± 1.0	30.4 ± 0.7	30.7± 0.50
	Bacillus subtilis ATCC 6633	40.7 ± 1.3	20.3 ± 0.89	40.8 ± 0.98
	Staphylococcus epidermidis Clinical isolate	48.0 ± 2.0	25.0 ± 0.8	25.0 ± 1.0
	Micrococcus Luteus Clinical isolate	45.0 ± 1.0	32.0 ± 2.0	34.0 ± 0.7
Gram Negative Bacteria	*Escherichia coli* ATCC 8739	0.0	0.0	0.0
	Klebsiella pneumoniae Clinical isolate	11.5 ± 0.4	15.0 ± 0.1	20.0 ± 1.0
	Pseudomonas aeruginosa ATCC 9027	0.0	20.3 ± 0.9	20.5 ± 1.0
	Salmonella typhi ATCC 14028	20.0 ± 0.5	0.0	20.3 ± 1.8
Fungi	*Candida albicanc Clinical isolate*	18.0 ± 0.2	15.0 ± 0.4	13.0 ± 0.7
	Aspergillus niger Clinical isolate	28.0 ± 0.1	25.0 ± 0.3	28.0 ± 1.0

The Cytotoxicity of active fractions

Studies have been conducted using the MTT assay to test the extracts of *Streptomyces Spororaveus* DA 4 against Hct-116 and Vero cells. According to **Figure 6**, Fraction C has the lowest IC50 value of 224.57 ± 5.82 among the three fractions of human colorectal cancer cell line Hct-116. Fraction B comes in second with an IC50 value of 238.82 ± 2.57, while Fraction A comes in third with an IC50 of 341.28 ± 1.69. On the other hand, **Figure 7** demonstrated that Fraction C is the most cytotoxic among the three fractions for Vero cells, with the lowest IC50 value of 368.99 ± 4.34, followed by Fraction B at 607.95 ± 4.66 and Fraction A at 614.63 ± 1.96.

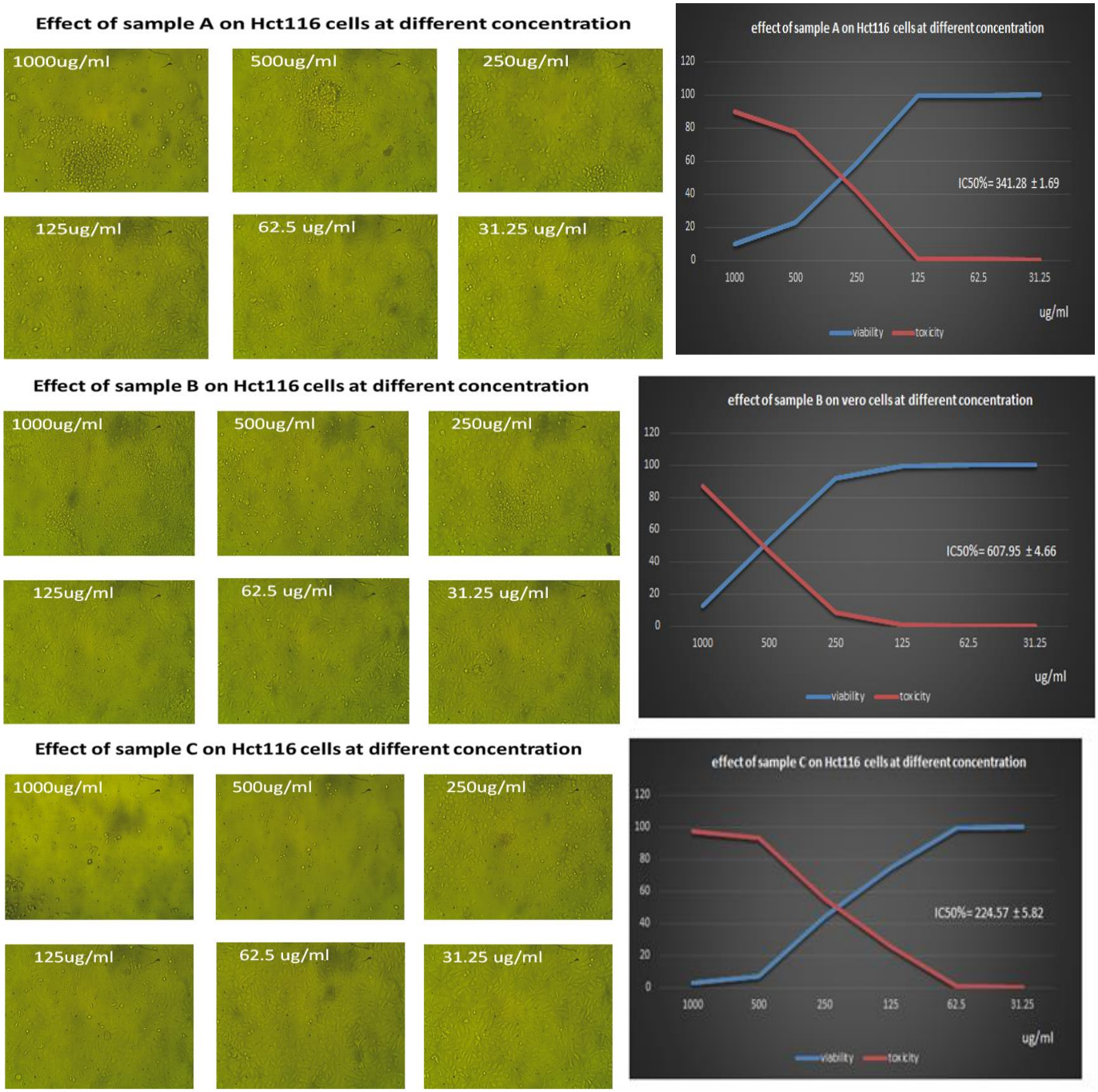


Figure 6 Cytotoxicity of Fraction A, Fraction B, and Fraction C against Hct116 cells respectively.

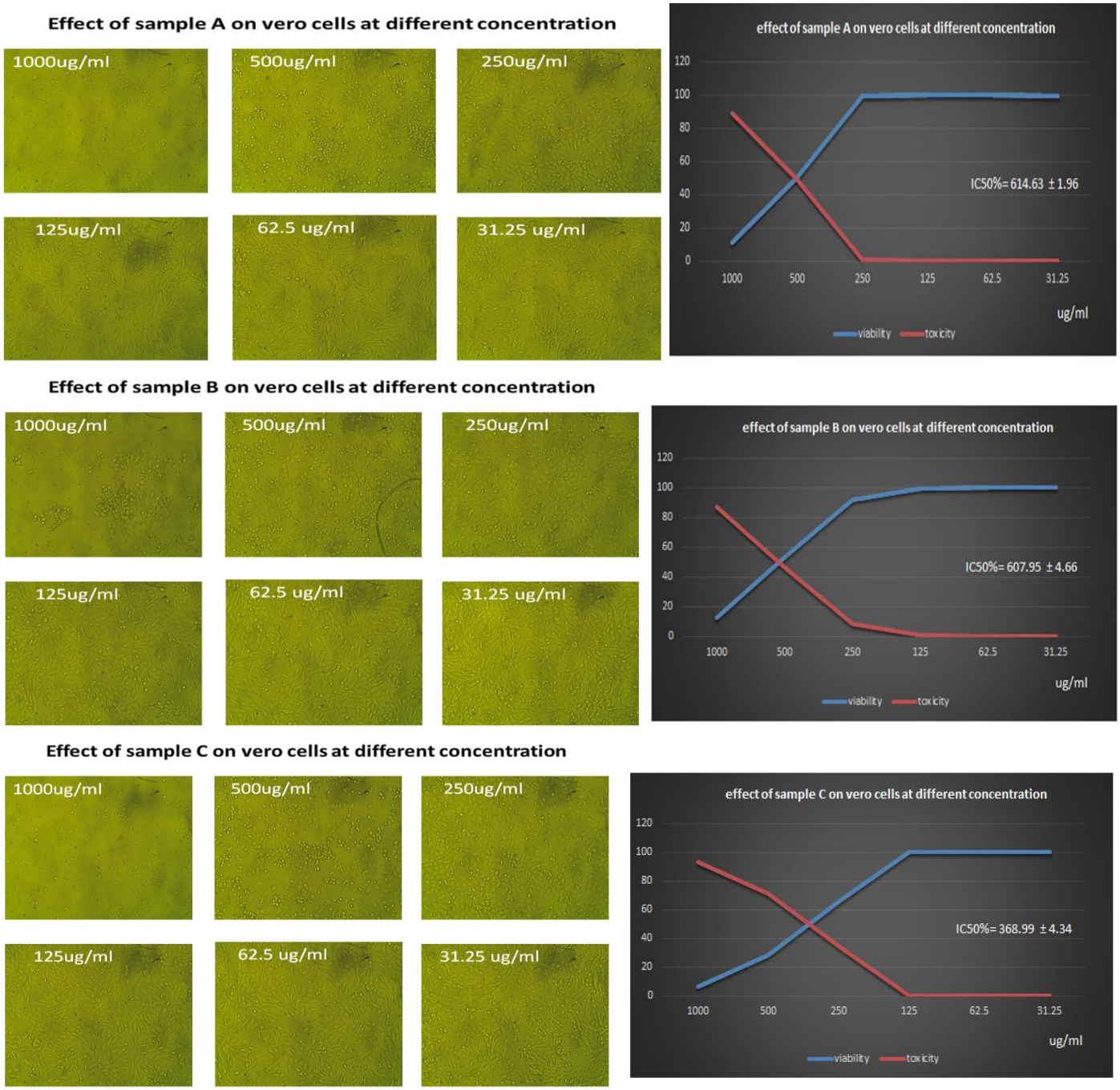


Figure 7 Cytotoxicity of Fraction A, Fraction B, and Fraction C on Vero cells respectively

DISCUSSION

Even with significant progress made in the identification of novel medication compounds, or antimicrobials, and the optimisation of their manufacturing processes, infectious diseases continue to rank as the second greatest cause of mortality globally **(De Lima ProcoÂpio *et al*., 2012)**. The emergence of multi-drug resistance in pathogenic bacteria is a primary cause of this, necessitating an ongoing search for novel and highly promising bioactive compounds. The good news is that bacteria continue to provide new pharmaceuticals. Microbes create secondary metabolites that are extremely valuable in both medicine and commerce. Actinomycetes are a class of microorganisms that are a never-ending supply of metabolites, including growth-promoting agents for plants and animals, antibiotics, and enzyme inhibitors.

Twenty strains of actinomycetes were obtained from the Saudi Arabian Yanbu region for this study. They demonstrate global antibacterial activity, as demonstrated by the outcomes of the cross-streak technique screening procedure against harmful microorganisms. In light of this, is in accord **(Egorov, 1985)**. Furthermore, the outcomes demonstrated that the DA5 strain had strong antibacterial activity against the test pathogens. This strain was therefore employed for more research.

Molecular approaches offer numerous benefits over conventional methods for the identification of microorganisms, including speed, labour-saving nature, sensitivity, specificity, and efficiency **(Lane, 1991; Adzitey *et al*., 2013; Wang and Salaza, 2016; Tan *et al*., 2018; Al-dhabi *et al*., 2016)**. By the phylogenetic relationship and 16S rRNA gene sequence analysis, the most potent strain, DN 4, was shown to belong to the same genus of *Streptomyces spororaveus* with 99%.

After being partially purified using HPLC, the supernatant revealed three pure active substances: A, B, and C. The composition of fractions A, B, and C was found to contain twelve, eighteen, and twenty-two compounds respectively. The majority of these substances are bioactive substances. For instance, the antibacterial and antioxidant activities (**Salama *et al*., 2022; Asghar and Choudahry, 2011)** of fatty acid and ester derivatives, such as 10-Octadecenoic acid methyl ester (46.90) and Hexadecanoic acid, 2, 3-dihydroxy propyl ester (23.51%), have been observed in Fraction A. Moreover, the presence of steroids such as 3-acetoxy (10.40%), 7,8-Epoxylanostan-11-ol, and ethyl 3,7,12-trihydroxycholan-24-oate [8.25%] has strong antibacterial action **(Salama *et al*., 2022)**, as well as antifungal and anti-anthracnose activity **(Alqahtani *et al*., 2020; Kaur *et al*., 2023)**. Fraction B contains a saturated fatty acid, n-hexadecenoic acid (palmitic acid) (30.56%), which affects Gram-negative bacteria **(Altieri *et al*., 2009; Farzaeia *et al*., 2014)** and can reduce the growth of harmful bacteria **(Shaaban *et al*., 2021)**. Furthermore, Octadecanoic acid, methyl ester (5.21%) **(Salama *et al*., 2022)** and Rhodamine 6G cation (5.03) have been shown to have harmful effects (**Kaur *et al*., 2023; Shaaban *et al*., 2021)**. 13-Docosenamide, (Z)-Erucylamide has also been shown to have antifungal and antibacterial action **(Zhang *et al*., 2018)**. Fraction C contains L-(+) ascorbic acid 2,6-dihexadecanoate. The previous literature also shows that the lipid derivatives including L-(+)-ascorbic acid 2,6-

dihexadecanoate which is characterized by marine sources and plants were proven to be a significant antibacterial, anticancer and antioxidant activity **(Karthikeyan *et al*., 2014)**. **Patil and Jadhav (2014)** validated the presence of Cycloheptasiloxane Tetradecamethyl in the GC-MS spectra of the leaf extract Toddalia asiatica (L.). Furthermore, **Manorenjetha et al. (2013)** revealed that Cycloheptasiloxane and tetradecamethyl had good antibacterial, antifungal, antifouling, immunomodulatory, and antitumor properties.

The three fractions A, B, and C, on the other hand, showed strong antibacterial activity against Gram (-ve) bacteria, Gram (+ve) bacteria, and fungi from the examined microorganisms. The results of our study align with the investigation conducted by **Sanghvi et al. (2014)**, which utilised thin-layer chromatography to evaluate the antibacterial capacity of two bioactive fractions extracted from *Streptomyces werraensis* ethyl acetate. Fraction A revealed the presence of twelve chemicals, accounting for 97.14% of the overall extract composition. 10-octadecenoic acid methyl ester, Hexadecanoic acid, 2, 3-dihydroxy propyl ester, 2-hydroxy-3-[(9E)-9-octadecenoyloxy] propyl (9E)-9-octadecenoate, 9-octadecenoic acid (Z)-2[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy] methyl] ethyl ester, octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, and methyl 3-oxooctadecanoate. All of these chemicals are fatty acid derivatives that were found in fractions. Hexadecanoic acid, often known as palmitate, has been shown to have antibacterial and antifungal effects **(Chandrasekaran *et al*., 2011; Rahuman *et al*., 2000)**. Furthermore, ethyl 3,7,12-trihydroxycholan-24-oate possesses antibacterial properties and can be employed as a synthesis intermediate for other medicines **(Mikov *et al*., 2007)**. Furthermore, Fraction B demonstrated the existence of 18 chemicals accounting for 95.32% of the total extract composition, among which Hexadecanoic acid, methyl ester, and n-hexadecanoic acid. Octadecanoic Acid, Methyl Ester Octadecenoic acid All of these chemicals are fatty acid derivatives that were found in fraction B. Hexadecanoic acid, often known as palmitate, has been found to have antibacterial and antifungal effects **(Asghar and Choudahry, 2011; Wei *et al*., 2011)**. Moreover, Fraction C demonstrated the presence of 22 chemicals accounting for 94.41% of the overall extract composition, including Trimethoprim (TMP), which provides antibacterial coverage against *E. coli*, as reported by **Nguyen et al. (2010)**. On the other hand, the presence of 10,13-Octadecadiynoic acid, methyl ester, and Butyl 9,12-octadecadienoate, as well as Oleic Acid 9-Octadecenoic acid, which exhibit antibacterial action according to **Asghar and Choudhary (2011); Chandrasekaran et al. (2011) and Wei et al. (2011)** who confirmed our results. Furthermore, glycidyl palmitate has been shown to have hypocholesterolemic, antibacterial, and antioxidant properties **(Arora *et al*., 2017)**.

As determined by the MTT assay, the crude extract exhibited cytotoxic activity against cell lines. The study found that Fraction C has the lowest IC50 value (224.57 ± 5.82), followed by Fraction B (238.82 ± 2.57) and Fraction A (341.28 ± 1.69) on HCT-116. The identification and characterization of a bioactive metabolite was precipitated by the extraordinary potential of the crude extract. The crude extract was fractionated and separated using a variety of chromatographic methods and spectroscopic studies. Also, save for HCT-116, all of the examined colon cancer cell lines have a defective p53 tumour protein. As a result, the three fractions' varying levels of cytotoxic activity against these colon cancer cell lines might be attributed to their p53 tumour suppressor protein status **(Petitjean *et al*., 2007; Goh et al., 2014)**.

On the other hand, the cytotoxic impact of fraction A may be attributed to the presence of 10-Octadecenoic acid methyl ester, Hexadecanoic acid, 2, 3-dihydroxypropyl ester, 2-hydroxy-3-[(9E)-9-octadecenoyloxy] propyl (9E)-9-octadecenoate, 9-octadecenoic acid (Z)-, 2-[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy] methyl] ethyl ester, Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, and Methyl 3-oxooctadecanoate. Previous research found that hexadecenoic acid caused cytotoxicity in human leukaemia cells at 12.5 to 50 µg/ml concentrations, whereas normal human dermal fibroblasts did not exhibit this behaviour **(Al-Wahaibi *et al*., 2020)**. Furthermore, the presence of Rhodamine 6G cation in Fraction B suppressed Consistent with previous research, both colon cancer and normal cell lines exhibited proliferation **(Lampidis *et al*., 1985)**. Also, cationic rhodamine dyes are good candidates for cytotoxicity on these cell lines, with findings reaching back as far as the 1970s **(Gear, 1974; Kurtoglu and Lampidis, 2009; Chen *et al*., 1982)**. The majority of these investigations show that cationic chemicals concentrate in tumour cells' mitochondria because tumour cells have an extremely high negative mitochondrial membrane potential as compared to normal cells. Cationic chemicals, when accumulated and then retained, disrupt adenosine triphosphate (ATP) synthesis in the mitochondria, removing the cells' fuel source **(Siddharth and Vittal, 2018; Cappuccino, 2002)**. Furthermore, Fraction C cytotoxicity is owing to the presence of trimethoprim (TMP). This is because TMP, folic acid antagonists, inhibit the activity of dihydrofolate reductase enzymes, which are responsible for converting dihydrofolate to tetrahydrofolate and so altering nucleotide production **(Mclean and Faed, 1990; Baccanari, 1995)**. A folate deficiency is now widely recognised as a significant factor in the development of cancer, and folate antagonists have been shown to induce the entire range of genetic alterations that are commonly linked to environmental geno-toxicants.

CONCLUSION

The current data provides valuable insight and is the first study on the biological activities of *Streptomyces spororaveus* DA 4. The bioactivity fractions A, B, and C were obtained using HPLC. The GC-MS examination was conducted to predict the structure of the three fractions. The isolated strain's supernatant contains a variety of fatty acids and bioactive fractions, including 10-octadecenioc acid, methyl ester, hexadecanoic acid, 2, 3-dihydroxy propyl ester, and 2-hydroxy-3-[(9E)-9-octadecenoyloxy] propyl (9E)-9-octadecenoate; 9-octadecenoic acid (Z)-2[(trimethylsilyl) oxy]-1-[[(trimethylsilyl)oxy] methyl] Ethyl ester, Ethyl 3,7,12-trihydroxycholan-24-oate, n-Hexadecanoic acid, Glycidyl palmitate, Methyl-3-oxooctadecanoate, Trimethoprim, and Tridecanedial were found to be effective inhibitors of a variety of infections. The fractions A, B, and C exhibited strong cytotoxicity against Hct-116 cell lines. The study reveals that *Streptomyces spororaveus* DA 4 is a rich potential source of several bioactive chemicals. Based on the findings, we believe that *Streptomyces spororaveus* DA 4 isolated from Yanbu City, Saudi Arabia, could be highly useful in future pharmacological and therapeutic applications.

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