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Purification and analysis of secondary metabolites from actinomycetes isolated from red soil: Insights into their therapeutic application

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Abstract

Actinomycetes, isolated from soil can produce diverse secondary metabolites with valuable therapeutic applications. This study aimed to isolate actinomycetes from red soil and evaluate their antibacterial, antioxidant, and biofilm-forming potential. Selected isolates exhibited significant antibacterial activity against Gram-positive bacteria in agar well diffusion assays. Further chemical characterization of their secondary metabolites using spectroscopic techniques confirmed the presence of functional groups responsible for their bioactivity. Antioxidant activity was assessed using free radical scavenging assays, revealing strong antioxidant capacity. Additionally, the isolates demonstrated notable biofilm-forming ability, essential for their survival in harsh environments, and may contribute to antibiotic resistance. These soil-derived actinomycetes' combined antibacterial and antioxidant properties highlight their potential as sources of natural compounds for pharmaceutical applications. The findings support the need for further studies on these bioactive compounds' toxicity, formulation, and clinical relevance. This research contributes to the growing interest in natural product discovery and provides promising candidates for developing alternative antimicrobial and antioxidant agents from red soil actinomycetes.

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Introduction

Actinobacteria are a highly diverse group of Gram-positive bacteria that play a vital ecological role, particularly in soil environments, where they decompose complex organic materials such as lignin and cellulose. Their high GC content is a distinctive genetic trait that enhances their stability and survival in harsh conditions (Jagannathan *et al*., 2021).

Morphologically, their filamentous, mycelium-like structures resemble those of fungi, allowing them to form extensive networks that facilitate colonization of varied substrates.

These features, combined with their adaptability to a wide range of environments—including acidic, alkaline, and nutrient-deficient soils make them ecologically significant and biotechnologically valuable (Ebency *et al*., 2024). One of the most studied genera within this group, *Streptomyces*, is renowned for its production of bioactive secondary metabolites, including antibiotics like streptomycin, tetracycline, and erythromycin. Collectively, actinobacteria produce a range of therapeutically and industrially important compounds such as antifungals, immunosuppressants, and enzymes, making them a major focus in drug discovery and bioremediation research. This study aims to isolate actinomycetes from red soil and investigate their antibacterial, antioxidant, and biofilm-forming potentials. Antimicrobial activity in actinomycetes stems from compounds that interfere with vital bacterial processes, including cell wall formation, protein synthesis, and nucleic acid replication. For example, certain antibiotics inhibit peptidoglycan synthesis, while others block ribosomal function or DNA replication, effectively controlling or eliminating pathogenic bacteria. Some metabolites also target bacterial enzymes, disrupting essential metabolic pathways and contributing to their role in addressing antibiotic resistance. Beyond antimicrobial properties, actinomycetes are known for producing natural antioxidants, such as phenolic acids and flavonoids that neutralize free radicals and reduce oxidative stress, which is implicated in diseases like cancer and neurodegeneration (Nithyalakshmi *et al*., 2025). These antioxidant compounds are also explored for their potential applications in medicine, food preservation, and environmental sustainability. Additionally, actinobacteria are capable of forming biofilms—structured microbial communities enclosed in an extracellular matrix made of polysaccharides, proteins, and nucleic acids that protect from environmental stressors and antimicrobial agents (Vaijayanthi *et al*., 2012). While biofilm formation enhances environmental resilience, it also poses a clinical challenge by contributing to persistent infections on medical devices and tissues due to their increased resistance compared to free-floating bacterial cells. Overall, the biochemical diversity and ecological resilience of actinomycetes, particularly those from red soil, highlight their immense potential in the search for novel antimicrobial agents, antioxidants, and strategies to manage biofilm-associated infections.

Materials and methods

Soil sample collection

The soil (red) sample was aseptically gathered from the Bharathidasan University region, located in Trichy, Tamil Nadu, India. The samples were collected from a depth of 10 to 15 cm beneath the soil surface (Babu *et al*., 2025).

Isolation of actinomycetes

To isolate and enumerate actinomycetes from soil samples, a serial dilution method was done using the spread plate technique. 1 gram of soil was mixed with 9 mL of distilled water and diluted in a series up to 10^8 dilutions. A 0.1 mL portion of each diluted sample was then plated on starch casein nitrate (SCN) agar medium at 30°C for 5 to 7 days (Babu *et al*., 2025). This was followed by sub-culturing on fresh SCN agar plates.

Screening of antimicrobial activity

Test bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Vibrio cholerae*, *Staphylococcus aureus*, and *Salmonella typhi* were inoculated in nutrient broth and incubated at 37°C for 24 hours to confirm growth. Isolated actinomycete strains were cultured in starch casein nitrate broth at 28°C for 7 days, and the cell-free supernatant was obtained by centrifugation at 8000 rpm for 12 minutes. Antibacterial activity was assessed using the agar well diffusion method on Muller-Hinton agar inoculated with test organisms.

Wells were filled with 100 µl of the supernatant, and plates were incubated at 37°C for 24 hours (Dhaini *et al*., 2025). Inhibition zones were measured and compared to streptomycin (positive control) and sterile distilled water (negative control) to evaluate antibacterial efficacy.

Identification of potential actinomycetes isolates

The isolates were classified based on their morphological features, which included the appearance of colony characteristics on the culture plate, the structure of both aerial and substrate hyphae, spore morphology, and the production of pigments. The purified actinomycete isolates were maintained on starch casein nitrate agar medium and glycerol stocks stored at -20°C for long-term preservation (Babu *et al*., 2025).

Extraction of secondary metabolites

a) Mass multiplication and solvent extraction

A potential culture of the selected isolates was inoculated into a 250 ml conical flask containing 200 ml of starch casein nitrate liquid broth. The cultures were incubated at 28°C for 7-10 days. After incubation, the actinomycetes broth was extracted using ethyl acetate (1:1 V). The solvent-treated supernatant was transferred to a separating funnel and shaken vigorously for 4 hours (Rammali *et al*., 2024). Following shaking, the organic phase was separated from the aqueous phase. The organic phase was then evaporated and resuspended in 1 mL of methanol before being transferred to a glass vial for further analysis.

Antibacterial assay of secondary metabolites

Test microorganisms, including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Vibrio cholerae*, *Staphylococcus aureus*, and *Salmonella typhi*, were inoculated into the nutrient broth using a sterile loop. The inoculated tubes were then incubated at 37°C for 24 hours, after which visible turbidity was observed, indicating microbial growth. Muller-Hinton Agar medium was sterilized and poured into sterile Petri dishes. The pathogens *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio cholerae* were swabbed onto the surface of the medium (Prastya *et al*., 2025). A cork borer was used to create wells in the agar. The samples were centrifuged at 5000 rpm for 10 minutes, and the supernatant was carefully transferred into fresh test tubes, while the pellet was discarded. A volume of 100 µL from each sample was added to the wells. Streptomycin was used as the positive control, and sterilized distilled water served as the negative control. The plates were incubated at 37°C for 24 hours. After incubation, zones of inhibition were observed around the antibiotic (streptomycin) and the sample (RSA1, RSA3), indicating antibacterial assay.

Determination of antioxidant activity

a) DPPH activity

The free radical scavenging activity of the extract was assessed using the DPPH radical scavenging assay. This method evaluates the hydrogen atom donation ability of the extract by observing the decolorization of a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH in methanol produces a violet/purple color, which fades to yellow or orange in the presence of

antioxidants (Smati *et al*., 2025). A 0.1 mM DPPH solution in methanol was prepared, and 1 mL of this solution was mixed with 3 mL of the extract (at varying concentrations of 20-100 µg/mL) in methanol. The reaction mixture was vortexed thoroughly and incubated in the dark at room temperature for 30 minutes. The absorbance of the resulting mixture was measured at 517 nm using a spectrophotometer. The percentage of DPPH radical scavenging activity was calculated using the following equation.

% DPPH radical scavenging activity = $\{(A0 - A1) / A0\} \times 100\%$

Where A0 represents the absorbance of the control, and A1 represents the absorbance of the extract or standard. The percentage of inhibition was then calculated based on these values.

Determination of biofilm activities

a) Tissue culture plate method

The isolates from freshly sub-cultured plates were inoculated into Starch Casein Nitrate broth supplemented with 8% glucose and incubated at 37°C for 24 hours under stationary conditions. After incubation, the culture was diluted to a 1:100 ratio. Each well of a sterile 96-well flat-bottom polystyrene microtiter plate was filled with 200 µL of the diluted culture. As a negative control, 100 µL of uninoculated media was also added to wells in triplicate. The microtiter plate was incubated at 37°C for 48 hours. After the incubation, the wells were washed three times with 200 µL of phosphate-buffered saline (PBS) at pH 7.2. The biofilm formed on the plate was then fixed with 2% (w/v) sodium acetate for 10 minutes and stained with 0.1% (w/v) crystal violet for 10 minutes (Christensen *et al*., 2022). After staining, the wells were thoroughly washed with deionized water to remove any excess stain and then allowed to dry. To remove residual crystal violet, the stained polystyrene wells were rinsed twice with PBS. Upon air drying, the presence of a visible film along the walls and bottom of the wells indicated the production of biofilm.

b) Inhibition of biofilm on the glass tube surface

The Tube Method (TM) is a qualitative assay used to detect biofilm-producing microorganisms, based on the formation of a visible film. This method, as described by Christensen *et al.*, involves inoculating isolates into polystyrene test tubes containing Nutrient Broth. The tubes are incubated at 37°C for 24 hours (Deighton *et al*., 2020). After incubation, the sessile isolates that form biofilms on the walls of the polystyrene test tubes are stained with crystal violet for 1 hour, following the removal of planktonic cells by rinsing the tubes twice with phosphate-buffered saline (PBS). After staining, the test tubes are rinsed twice with PBS to remove any excess stain. The test tubes are then air-dried, and the presence of a visible film on the walls and bottom of the tube indicates biofilm production (Sahal *et al.,* 2015).

Characterization of potent bioactive compounds

a) Fourier transform infra-red spectrum

Fourier Transform Infrared Spectroscopy (FTIR) is a key tool for identifying functional groups in bioactive compounds by analyzing molecular vibrations across 4000–400 cm^{-1}, aiding in the preliminary characterization of actinomycete extracts (Johnson *et al*., 2025). This technique supports natural product research by linking functional groups like -OH, -C=O, and -NH to potential antimicrobial and antioxidant activities.

b) Gas chromatography- mass spectrometry

GC-MS separates and identifies bioactive compounds in actinomycete extracts by analyzing their volatility and mass spectra, with reference matching using databases like NIST or Wiley (Martinez *et al*., 2024). This technique is crucial in natural product research for detecting antimicrobial and antioxidant metabolites with therapeutic potential.

Results and discussion

Isolation of actinomycetes

In the present study, six actinomycete strains were isolated from a red soil sample, selected based on variations in colony morphology and color (Sharma *et al*., 2020). In the previous study, the colonies exhibited characteristics such as a tough, leathery texture, a dry or folded appearance, and a branching filamentous structure, with or without aerial mycelium, typical features of actinomycetes. The pure cultures of these soil actinomycetes were maintained on SCA (Starch Casein Agar) and stored at -20°C for further studies (Fig. 1 and Table 1).

Fig. 1. Subculture of Isolate of red soil actinomycetes

Table 1. Colony forming unit

Dilution factor	No. of colonies (CFU)
10^{-3}	38×10^{3} CFU
10^{-4}	28×10^{4} CFU
10^{-5}	15×10^{5} CFU
10^{-6}	10×10^{6} CFU

Screening of antimicrobial activity of actinomycetes

In our current study, antibacterial activity of the six isolates was assessed using the agar well diffusion method against seven different test organisms. After 24 hours of incubation, the zones of inhibition were measured and recorded. Two of the isolates were identified as potent strains, prompting further studies. The isolates RSA-1 and RSA-3 exhibited the largest zones of inhibition against *Klebsiella pneumoniae*, showed moderate activity against *Vibrio cholerae*, and displayed minimal activity against *Proteus vulgaris*. (Ahmed *et al*., 2021) also screened actinomycetes for antibacterial activity, reporting strong inhibition against *Klebsiella pneumoniae* and varying effects on other bacteria. Their study highlighted the effectiveness of the agar well diffusion method and reinforced the potential of actinomycetes as a source of bioactive antibacterial compounds (Fig. 2 and Table 2).

Table 2. Antibacterial activity of isolates

Bacterial pathogens	Isolated strains zone of inhibition (mm in diameter)						
	Control	RSA 1	RSA 2	RSA 3	RSA 4	RSA 5	RSA 6
Escherichia coli	32	+	-	+	-	-	-
Vibrio cholerae	32	+	-	+	-	-	-
Proteus vulgaris	32	+	-	+	-	-	-
Klebsiella pneumoniae	32	+	-	+	-	-	-
Staphylococcus aureus	32	+	-	+	-	-	-
Salmonella typhi	32	+	-	+	-	-	-

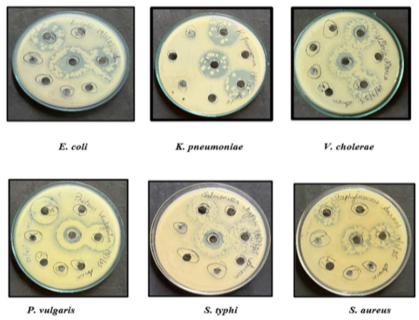

Fig. 2. Screening of antibacterial activity using agar well diffusion method

Cultural characterization

A loop full of culture was inoculated onto various media, including starch casein agar, ISP1 to ISP7, and incubated at 28°C for 7 days. The morphological characteristics, such as the color of aerial and substrate hyphae, were recorded following Shirling and Gottlieb's criteria (Ahmed *et al*., 2021). Both isolates were Gram-positive, with branched, filamentous hyphae and spore chains (Table 3 and 4).

Table 3. Cultural characterization for RSA-1 isolate

Morphology	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-6	ISP-7
Growth	Good	Good	Good	Not good	Good	Good	Good
Aerial mycelium	Half white	Half white	Golden color	No color	Sandal wood	white	Half white
Substrate mycelium	Half white	Half white	Golden color	No growth	Sandal wood	Half white	Sandal wood
Pigment Production				Nil			

Table 4. Cultural characterization for RSA-3 isolate

Morphology	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-6	ISP-7
Growth	Good	Good	Good	Not good	Good	Good	Good
Aerial mycelium	Half white	Half white	Golden color	No color	Sandalwood	white	Half white
Substrate mycelium	Half white	Half white	Golden color	No growth	Sandal wood	Half white	Sandal wood
Pigment production				Nil			

Biochemical characterization

Biochemical tests, including IMViC, urease, catalase, triple sugar iron, and oxidase tests, were conducted, with results presented in tabular form. The selected isolates, RSA-1 and RSA-3, showed positive reactions in the indole, methyl red, and oxidase tests. At the same time, they were negative for indole, Voges-Proskauer, citrate utilization, urease, and catalase.

Based on these biochemical characteristics, the isolates were identified as Actinomycetes species. (Singh *et al*., 2022) also used biochemical assays to identify Actinomycetes, emphasizing the importance of IMViC and oxidase tests in species differentiation. They noted that variations in citrate and urease tests indicate metabolic diversity, reinforcing the reliability of biochemical profiling for identification (Table 5).

Table 5. Biochemical characterization of RSA-1 and RSA-3 isolates

Biochemical tests	RSA-1	RSA-3
Vogues- Proskauer	-	-
Citrate utilization	-	-
Methyl red	+	+
Urease	-	-
Oxidase	+	+
Catalase	++	++
Triple sugar iron	-	-
Indole	-	-

(+) – Positive, (-) – Negative

Physicochemical characterization

Physiological tests were performed to characterize the actinomycetes, focusing on various characteristics such as the utilization of different nitrogen and carbon sources, as well as their tolerance to varying temperatures, pH levels, and salt concentrations. (Rao *et al*., 2021) also studied the physiological traits of Actinomycetes, emphasizing nitrogen and carbon source utilization in species differentiation. They highlighted the impact of temperature, pH, and salt tolerance on ecological adaptability, reinforcing the importance of physiological testing in Actinomycetes classification (Table 6).

Table 6. Physiochemical characterization of RSA-1 and RSA-3 isolate

Physiological characterization	Actinomycetes isolates	
	RSA - 1	RSA - 3
pH		
4	-	-
5	+	-
6	+	+
7	+	++
8	+++	+++
9	++	+
Temperature		
20°C	-	-
25°C	+++	+++
30°C	-	-
35°C	-	+
40°C	-	+
45°C	-	-
Carbon source		
Dextrose	++	++
Fructose	-	++
Glucose	+++	+++
Sucrose	++	+
Maltose	+++	++
Nitrogen source		
Alanine	+	+
Asparagine	+	+
Glutamine	++	-
Peptone	+	-
Yeast	+++	+++
Salt concentration		
0%	-	-
1%	+++	+

3%	+	+++
5%	+	++
7%	+	+++
9%	-	-

(+++ = Excellent, ++ = Moderate, - = no improvement)

Antibacterial assay of Secondary metabolites

The antibacterial activity of the crude extracts RSA-1 and RSA-3 was evaluated against bacterial pathogens *E. coli*, *S. aureus*, *V. cholerae*, *S. typhi*, *K. pneumoniae*, and *P. vulgaris*. Streptomycin was used as the positive control, while sterile distilled water served as the negative control. The isolates RSA-1 and RSA-3 exhibited the largest zones of inhibition against *Klebsiella pneumoniae and Proteus vulgaris*, showed moderate activity against *Vibrio cholerae*, and displayed minimal activity against *Salmonella typhi*. (Das *et al*., 2021) demonstrated that Actinomycetes-derived crude extracts exhibit strong antibacterial activity against both Gram-positive and Gram-negative bacteria, using streptomycin as a reference control (Fig. 3 and Table 7).

Table 7. Antibacterial assay of crude of RSA-1 and RSA-3

Isolated strain	Bacterial pathogens zone of inhibition (mm in diameter)					
	E. coli	*K. pneumoniae*	*S. aureus*	*S. typhi*	*V. cholerae*	*P. vulgaris*
Control	32	32	32	32	32	32
RSA-1	27mm	28mm	22mm	17mm	24mm	18mm
RSA-3	24mm	17mm	22mm	16mm	18mm	25mm

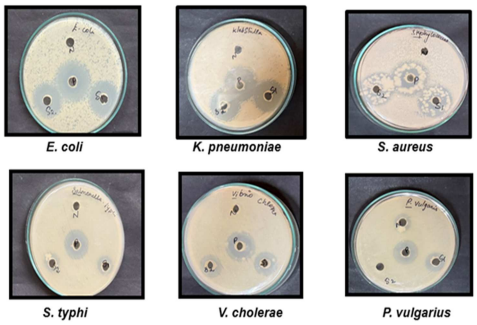

Fig. 3. Antibacterial assay of crude RSA-1 and RSA-3

Determination of antioxidant activity

a) DPPH free radical scavenging assay

In the present study, the antioxidant activity of crude extracts RSA-1 and RSA-3 was evaluated using the DPPH radical scavenging assay. Different concentrations of the crude extracts 980 µL, 960 µL, 940 µL, 920 µL, and 900 µL were tested. The reaction mixtures were incubated in the dark for 30 minutes to allow for decolorization from purple to yellow.

Absorbance was measured at 517 nm using a UV-Visible spectrophotometer, with methanol serving as the blank control. (Khan *et al*., 2021) reported that Actinomycetes-derived extracts exhibited strong, concentration-dependent antioxidant activity in the DPPH assay, effectively scavenging free radicals and indicating potential therapeutic value (Fig. 4 and 5).

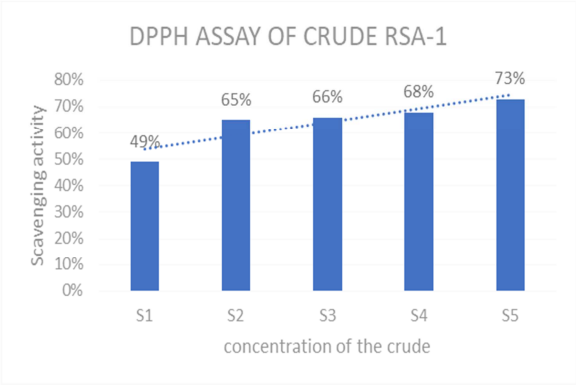

Fig. 4. DPPH Antioxidant activity of crude RSA-1

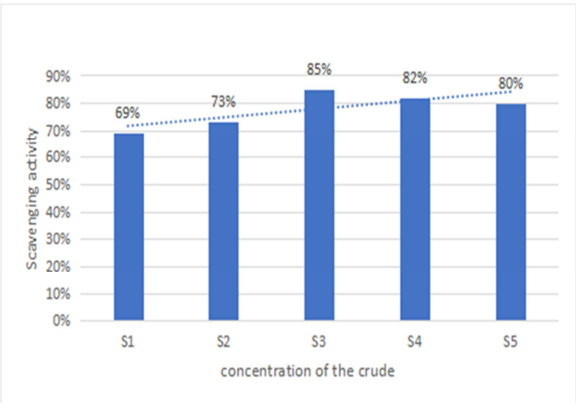

Fig. 5. DPPH Antioxidant activity of crude RSA-3

Determination of antibiofilm activities

a) Tissue culture plate method (TCP)

In this study, the anti-biofilm activity of extracts from soil actinomycetes was assessed against pathogens *E. coli*, *S. aureus*, *V. cholerae*, *S. typhi*, *K. pneumoniae*, and *P. vulgaris*. The isolates were inoculated into 96-well plates containing nutrient media and incubated at 37°C for 24 hours. After air drying and staining, the presence of a visible film on the walls and bottom of the well indicated biofilm formation, Results showed that the isolate RSA-1 showed moderate biofilm activity against *S. aureus* and *K. pneumoniae*, whereas the RSA-3 isolate showed the highest biofilm activity against *S. aureus*, *V. cholerae*, *K. pneumoniae*, and *P. vulgaris*, and moderate activity in *E. coli* and *S. typhi*. (Rao *et al*., 2021) Also studied the anti-biofilm properties of *Actinomycetes* extracts against bacterial pathogens, showing that their metabolites inhibit biofilm formation by preventing bacterial adhesion. The study highlighted their potential as alternative antimicrobials for controlling biofilm-associated infections.

b) Inhibition of biofilms on the glass tube surface

The potential actinomycete cultures, RSA-1 and RSA-3, were found to inhibit biofilm formation on the surface of glass tubes. These isolates effectively prevented the development of a biofilm ring on the glass surface. The selected isolates, RSA-1, demonstrated high inhibitory effects on biofilm formation by *E. coli*, *S. aureus*, *V. cholerae*, *S. typhi*, *K. pneumoniae*, and *P. vulgaris using* the method of crystal violet staining, and RSA-3 showed a less inhibitory effect on biofilm formation. (Mehta *et al*., 2022) demonstrated that actinomycetes-derived metabolites effectively inhibit biofilm formation by disrupting bacterial adhesion and matrix production, highlighting their potential in preventing biofilm-related infections.

Characterization of potent secondary metabolites

a) Analysis of FT-IR spectrum of crude extracts

The infrared spectra of the two strains, RSA-1 and RSA-3, revealed the presence of various functional groups, including halo compounds, sulfates, fluoro compounds, alkenes, aliphatic primary amines, alkanes, sulfonyl chlorides, and conjugated alkenes. A similar study by (Singh *et al*., 2021) analyzed the FTIR spectra of *Actinomycetes* isolates to identify functional groups associated with bioactive metabolites. Their findings confirmed the presence of key functional groups such as alkenes, amines, and sulfates, which play a crucial role in antimicrobial and antioxidant activity. The study emphasized FTIR spectroscopy as a valuable tool for characterizing secondary metabolites in *Actinomycetes*. These results further validate the potential of *Actinomycetes* in producing diverse bioactive compounds (Fig. 6).

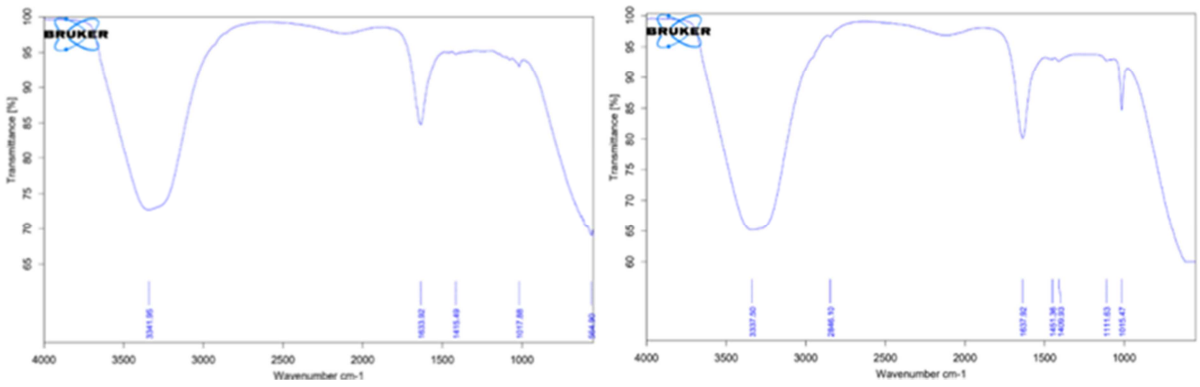

Fig. 6. FTIR spectrum of partially purified compounds of RSA-1 and RSA-3

b) GC-MS analysis of crude extracts

GC-MS analysis of the crude ethyl acetate extract identified 20 compounds in RSA-1, with major components including Cyclohexasiloxane, Octamethyl-, Benzoic Acid, 2,4-Bis (Trimethylsiloxy)-, Trimethylsilyl Ester, and 13-Docosenamide (Z),1,2-Benzenedicarboxylic Acid, Dibutyl Ester. These compounds are known for their antimicrobial, antifriction, anti-inflammatory, and antioxidant properties. Similarly, RSA-3 contained 15 chemical compounds, with key constituents such as Cyclohexasiloxane, Dodecamethyl-, 4H-Pyrrolo[3,2,1-IJ]Quinoline-2,9-Dione, Octahydro-6A-(2-Methoxyethyl)-, and 4H-Imidazol-4-One, 3,5-Dihydro-2-Methyl-3-(Methyl-D3)-5,5-Diphenyl.

Previous research has shown that 1,2-Benzenedicarboxylic Acid, Dibutyl Ester exhibits antioxidant and pesticide properties, while 9,12-octadecadienoic acid has been associated with anticancer and anti-inflammatory effects. A similar study by Kumar *et al*. (2022) identified multiple bioactive compounds with antimicrobial, antioxidant, and anti-inflammatory properties. The study emphasized the significance of GC-MS in identifying secondary metabolites with pharmaceutical applications. These findings further validate the potential of Actinomycetes as a source of bioactive compounds.

Conclusion

This study demonstrated the strong antibacterial potential of RSA 1 and RSA 3, showing significant activity against Gram-positive bacteria and suggesting interference with cell wall integrity and metabolic functions. Spectroscopic analysis confirmed key functional groups responsible for bioactivity, with results comparable to standard antibiotics. These findings highlight the potential of RSA 1 and RSA 3 as alternative antimicrobial agents for pharmaceutical applications. Further research on toxicity, formulation, and clinical efficacy is needed to support their therapeutic use.

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