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Research Article

Antagonistic and Antioxidant Potential of Actinomycete Isolates from Sambhar Lake

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Abstract: Bacterial secondary metabolites contain a diverse spectrum of biologically active compounds, including antimicrobials and antioxidants. Harnessing these products has enabled humans to fight against various diseases. But due to the overuse of antibiotics over the past few decades, many resistant strains of microbes are emerging. Therefore, there is a need to identify new sources of antimicrobial compounds. Actinomycetes are a group of bacteria known to produce antimicrobial compounds. This investigation aimed to identify, isolate and characterize actinomycetes with antimicrobial and antioxidant activity against pathogens acquired from MTCC. Sambhar Lake has a very potential group of actinomycetes producing several secondary metabolites. The study reports the antimicrobial and antioxidant potential of different extracts of actinomycetes. The sections of the actinomycetes were tested for their antagonistic activity against five pathogenic microorganisms. Antibacterial activity was reported in all five actinomycete isolates against *Bacillus subtilis*-MTCC 441, *Escherichia coli*-MTCC-1885, and *Salmonella typhi*-MTCC 733 but almost negligible activity against *Staphylococcus aureus*-MTCC 737 and *Pseudomonas aeruginosa*- MTCC 424 except for ACT-14 and ACT-11. ACT14 had the highest antibacterial activity compared to all other isolates. The antioxidant capacities of all isolated extracts were evaluated for total phenols, total antioxidant activity, total reducing power, DPPH, and ABTS assays. Section of *Pseudonocardia Antarctica* ACT14 showed the highest (23.89±0.96) free radical scavenging of 2, 2-diphenyl1-picrylhydrazyl (DPPH), phenolic activity (194 ± 6.05) activity. This study was conducted to identify novel actinomycetes with antimicrobial properties. The sample ACT14 was found to be a novel actinomycete (*Pseudonocardia Antarctica*), and this isolate showed antimicrobial activity against all the test pathogenic bacteria used in the study. The culture was optimized for antimicrobial compound production. Since many pathogens tend to acquire antibiotic resistance, this study is essential as a novel source of antimicrobials that will provide a new weapon in the fight against infectious pathogens. Further purification and characterization of this compound are underway.

Keywords: Antimicrobial Activity, Multidrug Resistance (MDR), ABTS Assay, and DPPH Assay.

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1. INTRODUCTION

Increasing antibiotic resistance around the globe is an area of concern. Due to the emergence of new drug-resistant strains, many existing antibiotics are becoming ineffective against bacterial and fungal infections. It is estimated that if further interventions are not introduced, more than 10 million people will die from antibiotic-resistant diseases by 2050[1]. As a result, there is an urgent medical need to seek out new antimicrobial agents derived from natural sources with novel mechanisms of action. However, even if novel interventions are implemented, the risk of developing novel multidrug-resistant pathogens remains in society[2]. Therefore, a constant search for new antibacterial compounds with vigorous antibiotic activity and novel mechanisms of action will be required[3].Novel microbial strains can be valuable sources of new bioactive substances with antibiotic properties[4]. The soil is an essential source of microbes and a heavily exploited niche in which the microbial population produces many valuable natural products with biological activity, including clinically significant antibiotics. Actinomycetes have made many commercially beneficial bioactive compounds in recent decades and are routinely tested for new bioactive compounds. Actinomycetes are responsible for approximately two-thirds of all natural antibiotics[5, 6]. Actinomycetes were classified as gram-positive bacteria but are unique enough to be grouped into a separate category. They resemble fungi morphologically because of elongated cells with branches reaching the shape of filaments. These hyphae are abundant and smaller than fungal hyphae[6]. Actinomycetes are essential components of the bacterial community, particularly in stressed environments like high pH, high temperature, and water stress. From a biotechnological and economic point of view, these are considered the most critical prokaryotes, and they produce many important bioactive secondary metabolites and a variety of biologically active compounds[7, 8]. The biological compounds produced by Actinomycetes have been shown to possess antiviral, antifungal, antitumor, insecticide, antioxidant, anti-inflammatory, antifungal, antiparasitic, and immunosuppressive activities. Several herbicides, enzyme inhibitors, and critical industrial compounds have also been obtained from actinomycetes[9]. Advanced molecular tools, for example, metagenomics, metatranscriptomics, and metaproteomics can extract and study DNA, RNA, and proteins from ecological samples. It is now possible to use selective primers to amplify specific actinomycete 16S rRNA genes. The 16S rRNA amplification products from environmental samples can be sequenced to identify novel actinomycetes in environmental samples[10]. Extensive research has been conducted in various coastal areas of India to isolate and cultivate actinomycetes[10]. However, the Sambhar Salt Lake in Rajasthan, India, has been relatively unexplored. As a result, this study aimed to isolate and characterize actinomycetes from this geographical area. The molecular characterization and the phylogenetic comparison of isolates were to identify new actinomycetes. In addition, the isolates were screened for various functional characteristics such as antibacterial, antioxidant, and free radicals scavenging activity[11]. This study reports a novel actinomycete isolate ACT14 with potent activity against multiple pathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Soil samples (100 g) were collected from 5 different regions at Sambhar (Salt Lake) Jaipur, Rajasthan, India (26°55'12 "N and 75°12'00" E) at a depth of 15-20cm. Samples were packaged in sterile polyethylene bags under cooling and controlled conditions and transported aseptically for further analysis to the laboratory at the earliest[12].

2.2 Isolation of Actinomycetes

Samples were processed immediately for isolation of Actinomycetes (via serial dilution method) [13].1g of each sample was dispersed in 9 ml of sterile saline solution and stirred for 2 minutes. Next, the samples were heat-treated for 10 minutes in a water bath at 60°C to eliminate non-sporulated bacteria[14]. After dilution, the suspended solution was serially diluted to 10^{-7} in saline and 100 µl of diluted solution (10^{-3} to 10^{-7}) was individually distributed on actinomycete isolation agar containing 50µg/ml of nystatin to minimize contamination by fungus. At last, these plates were incubated at 28°C for 7-30 days, and Actinomycetes were screened based on colony morphologies[15].

2.3 Preliminary Screening for Antimicrobial Activity

The different isolates of actinomycetes were screened by the cross-streak method for biological activity against pathogens. In particular, Actinomycetes single streak was made on Mueller-Hinton agar medium (Himedia India) and incubated for 4 days at 28 ± 2°C[10]. After observing the good growth of the ribbon-shaped actinomycetes, pathogens such as *Salmonella Typhi* - MTCC 733[16], *Pseudomonas aeruginosa*- MTCC 424[17], *Escherichia coli*- MTCC 1885[16], *Bacillus subtilis*- MTCC441[18], *Staphylococcus aureus*- MTCC 737[19]acquired from the Microbial Type Culture Collection, Institute of Microbial Technology organism (IMTECH), Chandigarh, were streaked at right angles to the original actinomycetes streak and incubated at 37°C. The zone of inhibition was measured after 24 to 48 hours[20]. The actinomycetes isolates that showed antimicrobial activity against pathogenic bacteria were selected for further screening.

2.4 Optimization of Cell Growth for the Production of Bioactive Compounds

The selected isolates of actinomycetes were placed in three different flasks and incubated for 7 days at 30°C on a shaker in three various media: starch-casein media, actinomycetes isolation media, and glycerol asparagine media to maximize antibacterial production and cell mass[10]. After incubation, the cultures were screened for antimicrobial activity against the previously mentioned experimental pathogens by the good diffusion method[20].

2.5 Extraction of Bioactive Compounds

The antimicrobial activity of the strains isolated was tested against five different strains of bacteria, including *Pseudomonas aeruginosa*-MTCC 424, *Salmonella Typhi*-MTCC 733, *Escherichia coli*-MTCC 1885, *Bacillus subtilis*-MTCC 441, and *Staphylococcus aureus*-MTCC 737. The isolates were inoculated into a starch casein medium and shaken at 250 rpm for 7 days at 28 ± 2°C. The culture medium was centrifuged at 8000 rpm for 15 minutes after incubation, and the supernatant was collected. The supernatant was aseptically transferred to conical flasks, and an equivalent volume of five different solvents (chloroform, hexane, ethyl acetate, and methanol) was added separately to the cell-free culture filtrate and shaken continuously for two hours. The upper solvent layer was then

separated and transferred to a new flask. The solvent was dried out in a vacuum at 35^{0}C, and the remaining dried material was then scrapped and dissolved in DMSO (dimethyl sulfoxide) to a final concentration of 1mg/ml before testing for antibacterial activity using the good diffusion method [21].

2.6 Secondary Screening

The agar well diffusion method was used for secondary screening of actinomycetes based on the inhibitory effect of different extracts on the test organism[22, 23]. *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli, Salmonella typhi, and Staphylococcus aureus* were tested on Muller Hinton agar (Himedia, India). Wells (8 mm) were created using a sterile agar borer, and 40µl of each extract (1mg/ml) was added to each well separately. Ofloxacin dissolved in DMSO (1mg/ml) was used as a positive control. The plate was allowed to sit at room temperature for 20-30 minutes for the antibiotic fraction to diffuse before incubating it at 37°C for 24 hours. The zone of inhibition around the wells was measured in diameter. Each test was performed three times, and the antibacterial activity was calculated as the mean diameter of the inhibitory regions (mm) produced by the secondary metabolite [24].

2.7 Determination of Biological Activities of the Bioactive Compounds

2.7.1 ABTS Radical Scavenging Activity of Solvent Extracts

An ABTS assay was used to determine the effect of solvent extracts on the capture of ABTS free radicals[25]. The radical cation ABTS (2, 2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) is a blue chromophore formed by the reaction of ABTS and potassium persulphate. The blue chromophore of the ABTS•+ radical cation was reduced in the presence of actinomycetes extracts and quantified spectrophotometrically[26]. ABTS free radicals were created by reacting a 7mM ABTS stock solution with 2.45 mM potassium persulfate in the dark for 12 to 16 hours at room temperature. The solution was then diluted with distilled water to achieve an absorbance of 0.70 at 730 nm. After incubating 1 ml of different concentration (0-100Gg/ml) solvent extracts in 4 ml of ABTS free radical solution for an additional 30 minutes, the absorbance was measured at 730 nm. Ascorbic acid was used as a standard reference. The following formula was used for calculating free radical scavenging activity.

$$\text{Scavenging activity (\%)} = [(A-B) / A] \times 100$$

Where A denotes the absorbance of the ABTS solution without the extract / standard solution, and B indicates the sample's measured absorbance. The IC_{50} value, which represents the standard extracts concentration required for 50% free radical removal, was determined. For each sample the experiment was performed in triplicates.

2.7.2 DPPH Free Radical Scavenging Activity of Solvent Extracts

The DPPH assay, which is one of the most common and quick methods for determining the radical scavenging activity of biologically active extracts, was used [27]. A DPPH free radical scavenging test was performed to assess the radical scavenging activity of the Actinomycetes isolates solvent extract[25]. Actinomycetes extracts' antioxidant activity (AOA) was measured spectrophotometrically. As a measure of free radical scavenging activity, the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was used[28, 29]. 1 ml of DPPH (Sigma Aldrich) in methanol (5 mg/100 ml) was mixed with 0.5 ml of each properly diluted sample. After 30 minutes, the absorbance was measured at 517 nm. The results were given in mg/mL gallic acid (GA) equivalents. The experiment was performed in triplicates for each sample.

$$\%\text{Radical scavenging activity} = \frac{\text{Absorbance (control)} - \text{Absorbance(sample)} \times 100}{\text{Absorbance (control)}}$$

2.7.3 Determination of The Total Phenolics

Bioactive compounds' antioxidant activities are primarily due to their redox properties[27]. The antioxidant activity of phenolics is responsible for the antioxidant activity of the extracts. In addition, phenolic antioxidant activity is essential in absorbing and neutralizing free radicals[30]. The total phenol content of the five fractions (biological compounds) was determined by spectrophotometry using a slightly improved Folin-Ciocalteu reagent. The extracts were mixed with the Folin-Ciocalteu reagent (1:1) and 4 ml of sodium carbonate (1M) for 15 minutes. Using a spectrophotometer, the absorbance of extracts was measured at 765nm. The overall phenol content of the extract in µg gallic acid equivalent (GAE)/mg of the extract was estimated using various gallic acid concentrations (standard, 0-1000µg/ml) for drawing a standard curve. The experiment was repeated three times for each fraction, and average values of the results were used as final values[27].

2.7.4 Test Method for The Total Antioxidant Activity orPhosphomolybdenumAssay

The Phosphomolybdenum Assay test was used to determine the total antioxidant activity of extracts. 0.3 mL of each portion was mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In a water bath, the reaction mixture was incubated at 95°C for 90 minutes. All sample mixtures' absorbance at 695 nm was measured. Ascorbic acid (100µg/ml) served as a control[27]. Following formula was used to calculate % inhibition:

% Inhibition = (1- absorbance of the test sample / absorbance of the control) x 100

The experiment was performed in triplicates for each fraction.

2.7.5 Total Reducing Power

To determine the reducing power, the fraction containing the biologically active compound (100µg/ml) was mixed with 1% potassium ferricyanide and incubated for 20 minutes at 50°C. Next, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture and centrifuged for 10 minutes at 5000 rpm. Finally, the top solution (2.5 ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1 percent ferric chloride, and the colour development was measured at 700 nm[27]. The standard control was ascorbic acid (100µg/ml). The experiment was performed in triplicates for each sample.

2.7.6 Molecular Characterization of the organism

Genomic DNA was extracted from the highly active isolate (ACT14) and submitted to Yaazh Xenomics, Thabalthanthinagar, Madurai, for 16S rRNA sequence analysis[20]. The genomic DNA of ACT14 was used as a template, and universal bacterial primer pairs 27F (5 'AGAGTTTGATCMTGGCTCAG3') and 1492R (5 'AGAGTTTGATCMTGGCTCAG3') were used to amplify 16S rRNA over 30 cycles[31]. PCR reaction mixture consisted of 5 µL of isolated ACT14 DNA, 25 µL of PCR reaction solution (1.5 µL of forward and reverse primers, 5 µL of deionized water, and 12 µL of Taq master mix), 0.4mMdNTP, 3.2 mM$MgCl_2$ and Taq DNA polymerase. The PCR was carried out in the Eppendorf PCR Thermal cycler, and the Montage PCR purification kit was used to remove non-integrated PCR primers and dNTPs from the PCR products (Millipore).The ABI PRISM® BigDyeTM Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) was used for the sequencing reactions (Applied Biosystems). The 16S rRNA actinomycete sequence (ACT14), was deposited in NCBI. The NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST), was used to identify similar lines in 16s rRNA data based. Based on sequence similarity, the closest species to ACT14 actinomycete isolate was identified [32].

2.7.7 Phylogenetic Analysis

The NCBI BLAST tool was used to identify the similar 16S rRNA sequences in the database. The similar sequences were downloaded for further analysis. A phylogenetic analysis of the query sequence with the blast result's closely related sequences was performed[33]. Several lines are aligned using the MUSCLE 3.7 program[34]. Gblocks 0.91b was used to cure the resulting linear sequence. These Gblocks eliminate divergent regions (alignment noise suppression) and poor positions[35]. Finally, the aLRT program PhyML 3.0 was used for phylogenetic analysis, with HKY85 as a representative model. To render trees, the Tree Dyn 198.3 program was used[36].

3. RESULTS

3.1 Isolation and Characterization of Actinomycete Strains

Five different actinomycetes isolates were obtained from Jaipur's Sambhar Lake. The isolation of actinomycetes involves a crowded plate method. The isolates with significantly different morphology and pigmentation were purified by repeated streaking on a starch-casein-agar medium, Actinomycetes isolation agar and stored at 4°C as on slant. The chosen strain was aerobic, gram-positive bacteria. The colonies were dry, powdery, and fuzzy, with a concentric ring on the agar surface indicating secondary metabolite production and a diffusible white pigment initially identified as actinomycetes (Fig 1).

3.2 Preliminary Screening of Antimicrobial Activity

The isolated and purified Actinomycetes isolates were screened for antibacterial activity by cross streak method using Mueller-Hinton agar (Himedia, India) against five different pathogenic bacteria. Plates containing well-grown strains were Cross-streaked with pathogenic bacteria at a 90° angle and incubated overnight at 37°C. Antagonism was observed by detecting the absence or presence of the growth of pathogenic bacteria[10]. All isolates were screened for their antibacterial potential against five pathogenic strains. ACT-14 isolates showed the highest antibacterial activity based on the maximum inhibition of pathogenic strains, while ACT-10, ACT-11, ACT-13, and ACT-15 showed less activity against pathogenic microorganisms. Therefore, ACT14 was selected for further screening (Fig 2).

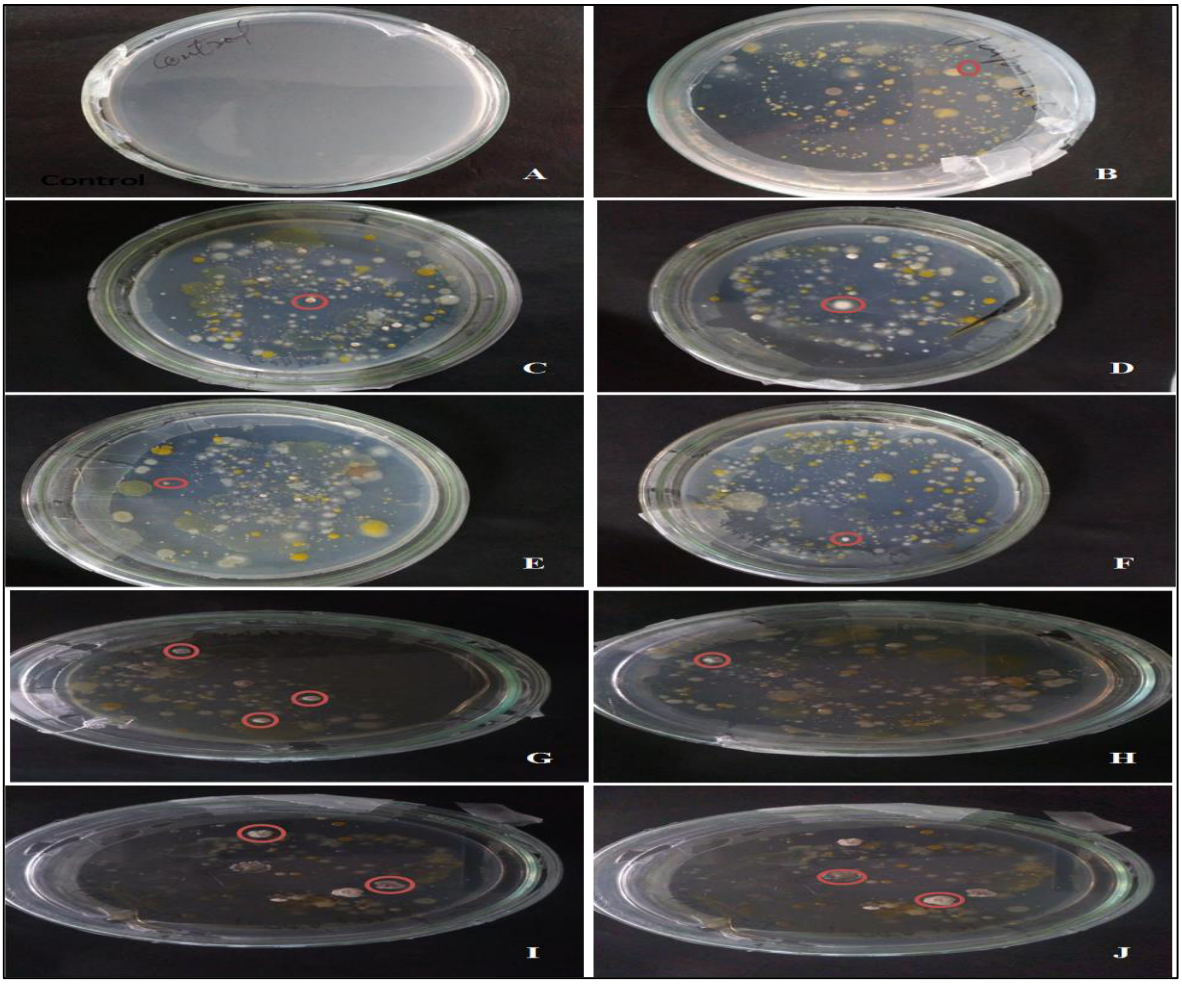

Fig 1: Isolation of Actinomycetes from Sambhar Lake soil samples by crowded plate method

The appearance of no growth around them encircled a total of 13 potential colonies. Actinomycete isolates, including slow-growing and rare actinomycetes, were isolated from Sambhar Lake soil samples using two different isolation media (Actinomycetes isolation agar and starch casein agar) with a prolonged incubation period. The crowded plate method facilitated the isolation of actinomycete strains, including members of some rare genera (*Pseudonocardia*). All the isolates were found to grow slowly on agar media.

3.3 Culture Media Study and Optimization of Cell Growth for The Extraction of the Compound

To maximize the antibacterial yield and cell mass, the strain ACT14 was cultured in three different media-starch casein (SC) media, actinomycetes isolation (AI) media, and glycerol asparagine (GA) media. The ACT14 isolate showed maximum antibacterial activity against all tested pathogens in the SC-medium as compared to other media. The glycerol asparagines broth extract had moderate antimicrobial activity. All other extracts showed minimal activity and the changes in the antimicrobial metabolite production among media may be attributed to the composition of the culture media in which the strain is grown. The ACT14 extracts showed maximum antibacterial activity against *Bacillus subtilis* and *Salmonella typhi*, with a zone of inhibition of about 16 mm against both. The response of ACT14 extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was lower, with a zone of inhibition of 10mm.

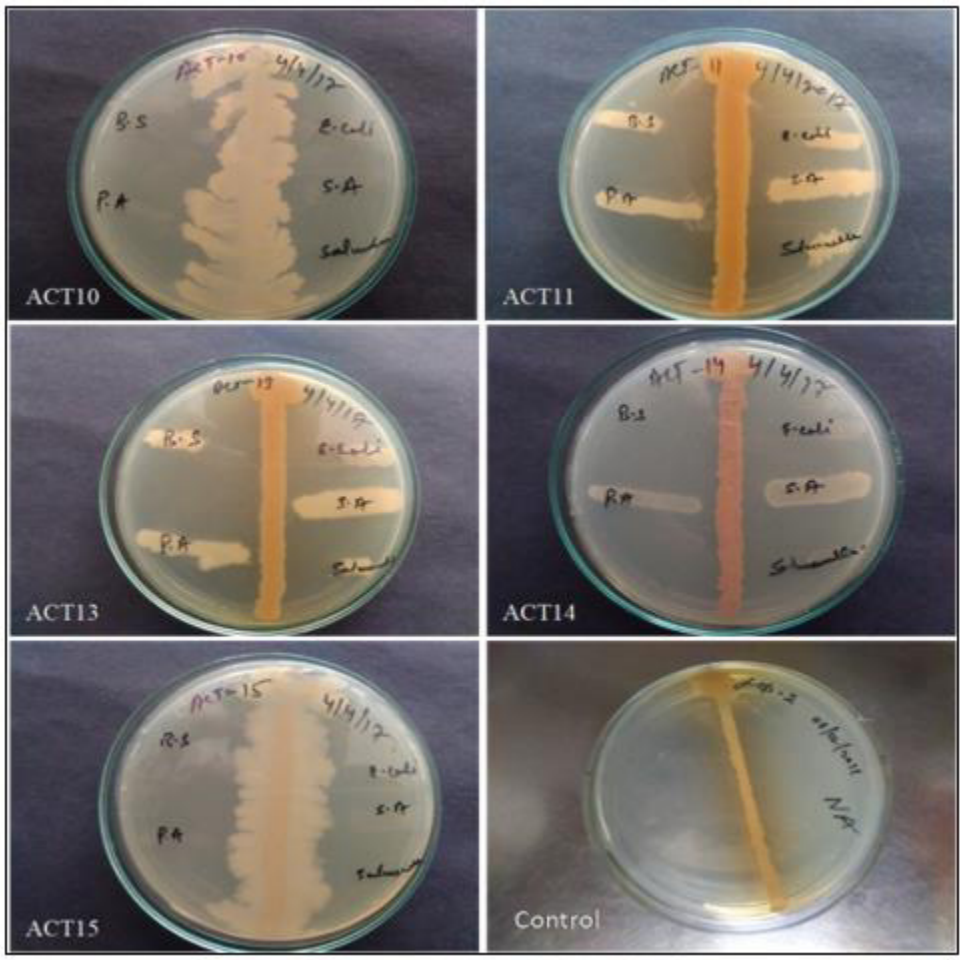

Fig 2: Antibacterial activity of extracts (ACT10, ACT11 ACT13 ACT14 and ACT15) in MHA media by cross streak method

The primary screening results of actinomycete samples against the pathogen used for the study are depicted. In primary screening, the cross-streak method screened actinomycete isolates for biological activity against pathogens. Actinomycetes single streak was made on Mueller-Hinton agar medium (Himedia India) and incubated for 4 days at 28 ± 2°C[10]. After observing the good growth of the ribbon-shaped actinomycetes, pathogens acquired from MTCC were streaked at right angles to the original actinomycetes streak and incubated at 37°C. The zone of inhibition was measured after 24 to 48hours. All samples showing activity against the pathogen used in the study were selected. The negative control used in the study showed no activity.

3.4 Secondary Screening of Antibacterial Compound

The bioactive compounds were extracted from the fermented broth using ethyl acetate as a solvent, and concentrated crude extract was used as a test compound against pathogenic strains *Salmonella Typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa* was carried out by agar well diffusion method. The result of antimicrobial activity of the different extracts against multidrug-resistant microbes as listed in Table no.1 and Fig.3 and using ofloxacin[18] as a positive control (susceptible against MDR samples).

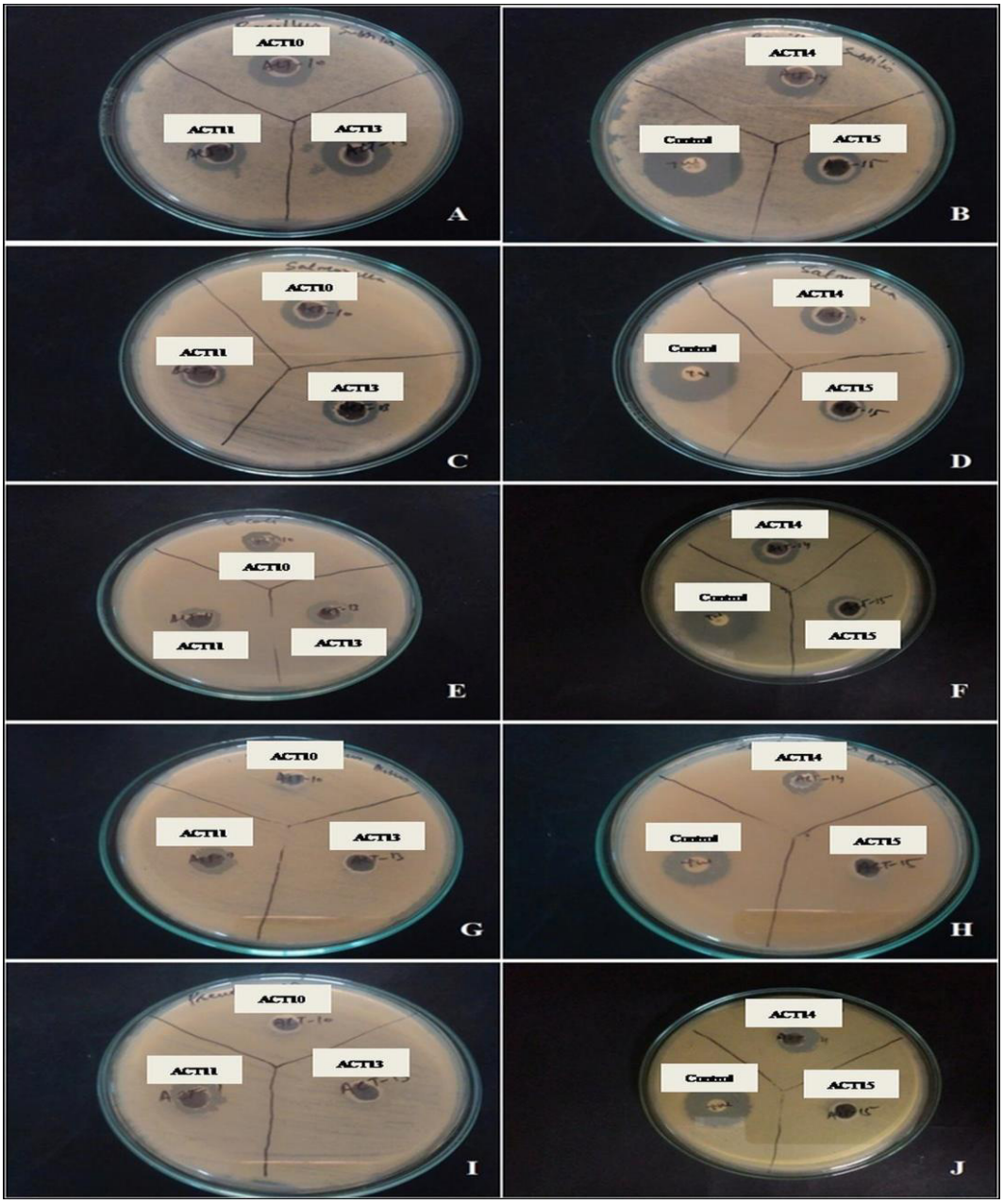

Fig 3: Agar well diffusion assays of the extracts. (A-B) against *Bacillus subtilis*;(C-D) against *Salmonella typhi*; (E-F) against *Escherichia coli*;(G-H) against *Staphylococcus aureus*; (I-J)against *Pseudomonas auroginosa*:

The agar well diffusion method for secondary screening of actinomycete isolates is depicted. The method utilizes the inhibitory impact of several extracts on the test organism. Ten plates were used, two for each culture. Using sterile agar borer, the wells were created, each pathogenic organism was swabbed on two different plates, and each extract was applied to each plate containing well individually for each culture. The plates were left at room temperature for 20-30 minutes to allow the antibiotic fraction to diffuse before being incubated at 37°C for 24 hours. The diameter of the zone of inhibition around the wells was measured in mm.

Table 1: Represents the antimicrobial activity of isolates (ACT-10, ACT-11, ACT-13, ACT-14, and ACT-15) against different MDR (multidrug-resistant) microbes

S. No	Isolates	*Bacillus subtilis* (in mm)	*Salmonellatyphi* (in mm)	*Escherichia coli* (in mm)	*Pseudomonas auroginosa* (in mm)	*Staphylococcus aureus* (in mm)
1	ACT10	6	7	6.5	-	-
2	ACT11	7	6.5	7	8	10
3	ACT13	16	7	7	-	-
4	**ACT14**	**16**	**16**	**14**	**10**	**10**
5	ACT15	10	7	6.5	-	-
'–' indicated the absence of an inhibition zone						

Table 1 illustrates the results of secondary screening. The extracts selected in primary screening were further screened for secondary screening by agar healthy diffusion method of actinomycete isolates. The technique utilizes the inhibitory

impact of several sections on the test organism. Extract of all actinomycetes was screened for their antimicrobial activity against each test microbe acquired from MTCC and plates were incubated at 37°C for 24 hours. After incubation the diameter of zone of inhibition of all the extracts was measured, ACT14 showed the best result among all the actinomycetes selected for secondary screening.

3.5 Morphological and Biochemical Characteristics of Selected Actinomycetes

All five isolates (ACT10, ACT11, ACT13, ACT14 and ACT15) selected in primary and secondary screening are from Sambhar Lake, Jaipur. The isolates with different morphology and pigmentation were purified by re-streaking on a starch-casein-agar medium, observed for morphological characteristics, and stored at 4°C (Table 2). The strains were gram-positive bacteria (aerobic), and the colonies were dry, powdery, and fuzzy, with a concentric ring on the agar surface indicating secondary metabolite production and a diffusible orange pigment that was initially identified as actinomycetes (Figure 4). A compound microscope was used to examine the strains, which revealed branched vegetative hyphae and aerial mycelium (Figure 5).

Table 2: Morphology characteristics of the selected actinomycetes isolates

Isolates	Medium	Diffusible pigment	Colony morphology	Aerial mycelium	Substrate Mycelium
ACT10	SCA	None	Sticky	White	White
ACT11	SCA	Orange	Rough	Light grey	Cream
ACT13	SCA	Orange	Sticky	Orange	White
ACT14	**SCA**	**Orange**	**Powdery**	**orange**	**White**
ACT15	SCA	None	Powdery	White	Cream

Table 2 illustrates the morphological characteristics of all the actinomycetes selected. The actinomycetes colonies often produce spores and pigment. The pigment production of all the actinomycetes was observed in starch casein agar. The colony morphology of all the actinomycetes was observed. Variation in colony morphologies across different actinomycetes was observed. ACT10 showed sticky morphology, ACT11 showed rough colonies, ACT14 & ACT15 showed a powdery appearance. Likewise, all the aerial mycelium and substrate mycelium were also observed.

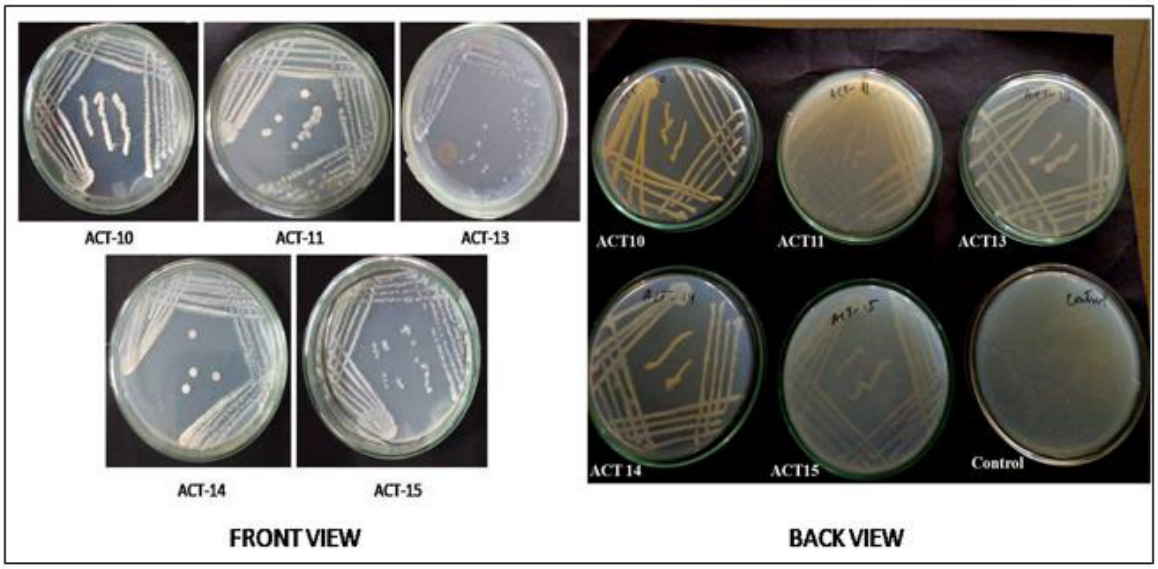

Fig4: Colony morphology of the selected actinomycete isolates on Starch casein agar (SCA) medium

The front view and back view of actinomycetes samples are depicted. The front view of the actinomycetes sample showed the colony morphology- aerial or substrate mycelium, while the back view often showed the diffusible color pigments of the actinomycetes sample. The colonies were dry, powdery, and fuzzy, with a concentric ring on the agar surface indicating secondary metabolite production and a diffusible orange pigment.

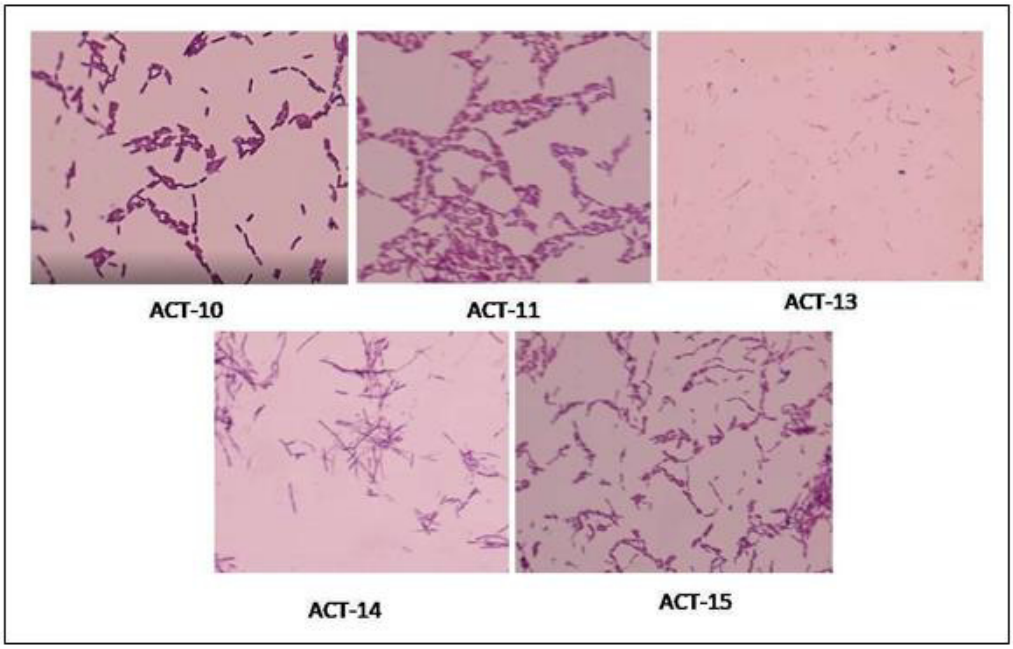

Fig 5: Gram staining and microscopic examination of the selected isolates of actinomycete

All the isolated strains were gram-positive bacteria (aerobic). The International *Streptomyces* project recognized and classified *Streptomyces* in to three major types: straight to flexuous (Rectiflexibles); spirals with one to two turns or hooks, loops (Retinaculiaperti); and spirals (Spirales). Strains in the current investigation also had spore chains that fit into one of the three categories identified. For example, spore chains of strains ACT10 and ACT13 were spirales, spore chains of strains ACT11 and ACT15 were rectiflexibles, and spore chains of strain ACT14 were retinaculiaperti.

3.6 Determination of Biological Activities of the Bioactive Compounds

3.6.1 Radical Scavenging Activity by ABTS

As the concentration increased, the antioxidant activity of all five extracts showed a similar increasing values. The ABTS+ radical cation scavenger activities of all samples at a concentration of 10 µg/ml were ACT-10 (26.53 ± 1.84), ACT-11(28.74 ± 1.55), ACT-13 (16.87 ± 2.33), ACT-14 (35.13 ± 0.13) and ACT-15 (45.43 ± 0.03). The maximum percentage of ABTS + free radical cation scavenger activity was 45.43 ± 0.03 compared with standard (48.51 ± 3.38) ascorbic acid. The IC_{50} of ABTS+ free radical cation scavenger activity of all five extracts was ACT-10 (37.63)µg/mL, ACT-11(26.02) µg/mL, ACT-13(31.54), ACT-14(24.46) and ACT-15(7.06) µg/ml (Figure 6).

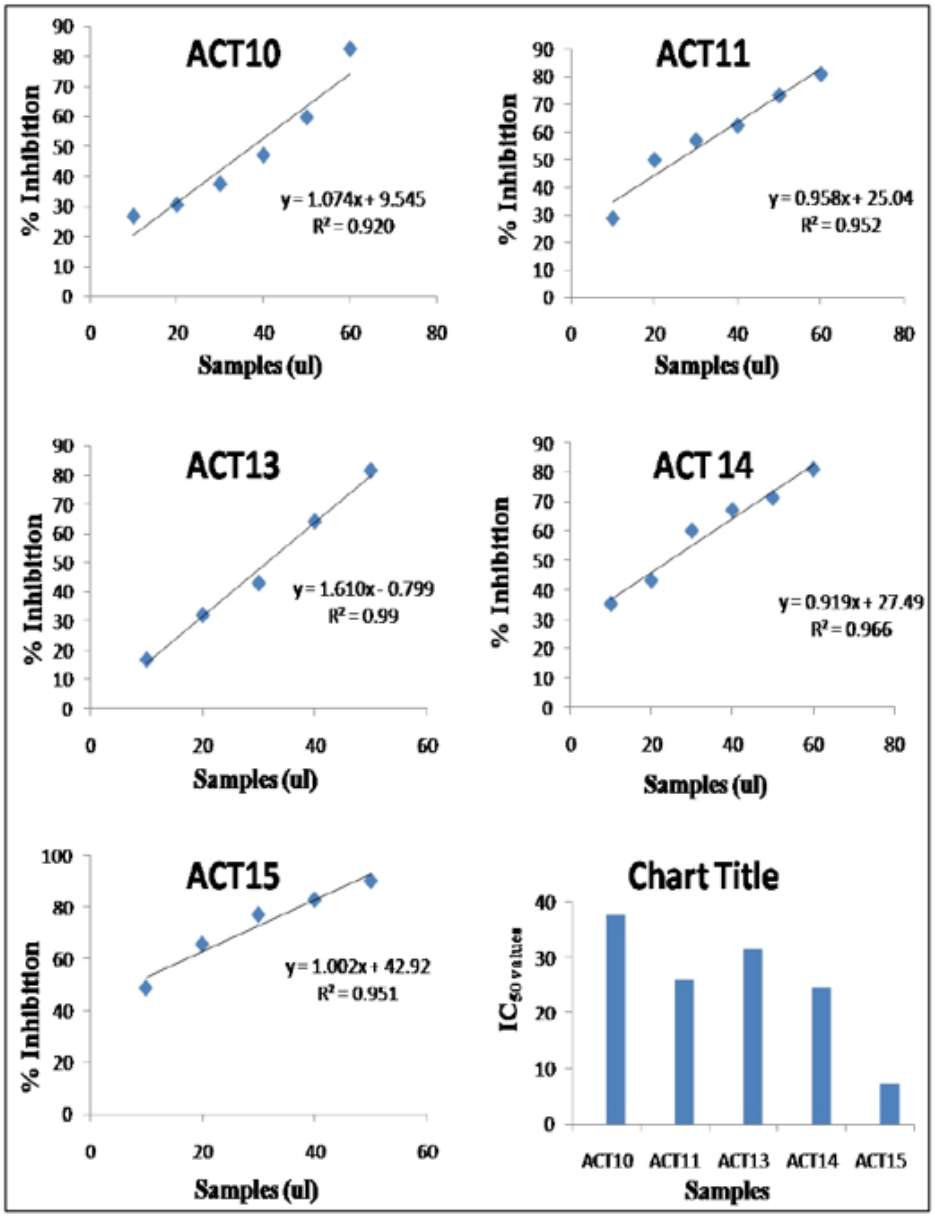

Fig 6: Antioxidant capacity of the culture extracts (ACT-10, ACT-11, ACT-13, ACT-14, and ACT-15), as per ABTS+ radical assay expressed as percentage activity and The IC_{50} of ABTS+ radical cation scavenging activity of all five extracts.

Antioxidants protect cells and organisms from oxidative stress by neutralizing ROS (reactive oxygen species), which causes oxidative damage. Antioxidants use a wide range of mechanisms, including scavenging free radicals, interfering with the auto-oxidation chain reaction, transforming reactive oxygen species (ROS) into stable molecules, and chelating metal prooxidants. The ABTS test uses a single electron transfer process. This method of the antioxidant test assesses extract's capacity to neutralize radical indicators via various mechanisms such as electron transfer or hydrogen transfer. In general, these quick and easy tests are used as preliminary screening to determine the antioxidant capacity of the extract. Given the absence of ABTS radicals in biological systems, separate sections were examined for high polyphenol content activity.

3.6.2 Radical Scavenging Activity by DPPH

The fractions (ACT-10, ACT-11, ACT-13, ACT-14, and ACT15) were able to scavenge DPPH (free radicals) and convert them into DPPHH. The active phytochemicals in the extract reduce the purple-coloured stabilizing (DPPH) 1, 1-diphenyl-2-picrylhydrazyl radical to yellow-colored 1, 1-diphenyl-2-picrylhydrazine, and this ability increases with increasing concentration[37]. Mix 1 ml of DPPH in methanol (5mg/100ml) with 0.5 ml of each appropriately diluted sample (100µg/ml). After 30 minutes, the absorbance at 517nm was measured. The results were expressed as mg/ml gallic acid equivalent (GA). The antioxidant activity (AOA) of all samples measured using the DPPH test is between 12.8 ± 1.3 (ACT-13) and 23.89±0.96 (ACT-14), expressed in gallic acid equivalents. The free radical scavenging effects of other samples were ACT-10(17.98 ± 1.23), ACT-11(13.70 ± 0.75), and ACT-15(14.44± 0.85) gallic acid equivalents (Figure 7).

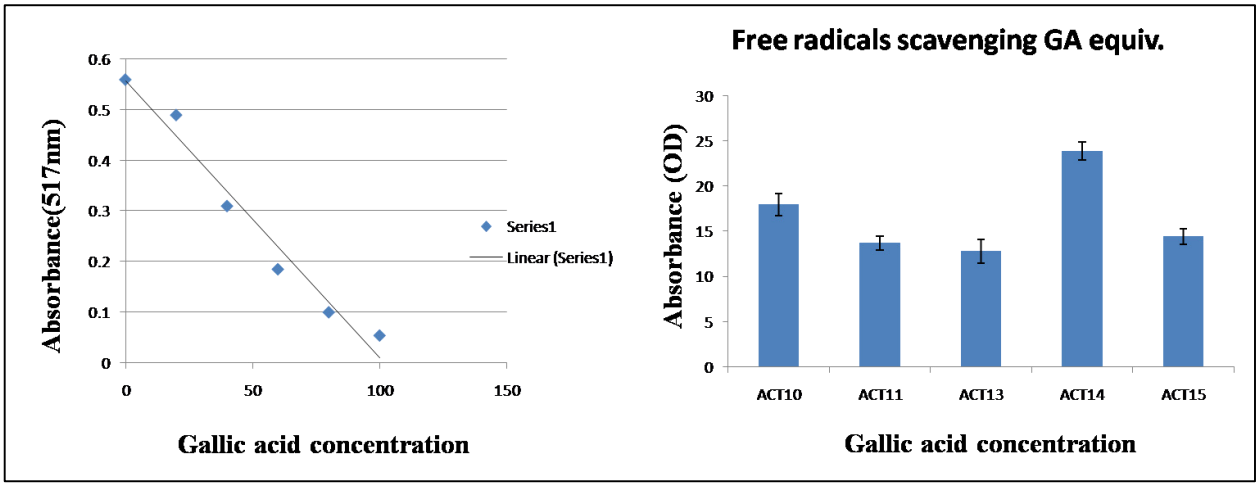

Fig 7: Free radical scavenging activity of five extracts and antioxidant activity measured with DPPH (mg/mL of gallic acid equivalents) assays.

The scavenging activity of DPPH is based on one-electron reduction which represents the free radical-reducing activity of antioxidants. Ascorbic acid (AA), quercetin (Qc) and gallic acid (GA) were used as positive control. All the samples were run in triplicates. Antioxidants protect cells and organisms from oxidative stress by neutralizing ROS (reactive oxygen species), which causes oxidative damage. Antioxidants use many mechanisms, including scavenging free radicals, interfering with the autoxidation chain reaction, transforming reactive oxygen species (ROS) into stable molecules, and chelating metal prooxidants. The DPPH test uses a single electron transfer process. This method of the antioxidant test assesses the extracts capacity to neutralize radical indicators via various mechanisms such as electron transfer or hydrogen transfer. In general, these quick and easy tests are used as preliminary screening to determine the antioxidant capacity of the extract. In addition, given the absence of DPPH radicals in biological systems, separate extracts were examined for high polyphenol content activity.

3.6.3 Determination of The Total Phenolics

The phenol content of each fraction is estimated to be be123.67 ± 5.00; 42.75 ± 0.90; 190.92 ± 6.03; 194 ± 6.05 and 50 ± 1.75 µgGA/g (ACT-10, ACT-11, ACT-13, ACT-14, and ACT-15), which is very similar to the phenolic activity of the standard (ascorbic acid) (194.86 ± 1.37µgGA/g) shown in Figure 8. The high total polyphenol contents can be attributed to biologically active compounds having the highest antioxidant capacity. The presence of phenolic groups in secondary metabolites is thought to be an important factor in antioxidant activity.

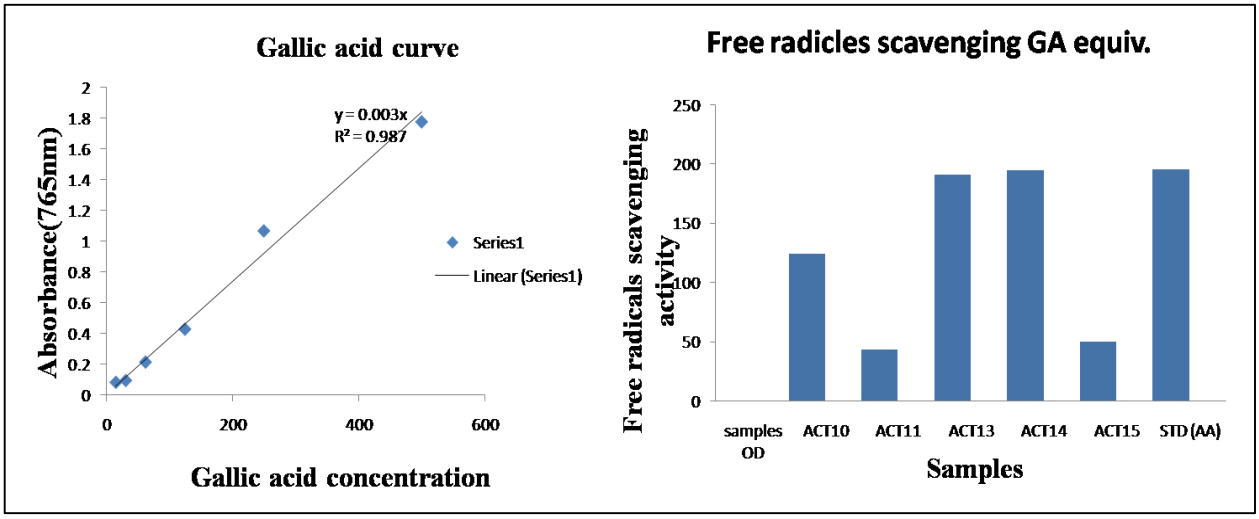

Fig 8: Variation of antioxidant capacity of different actinomycetes extract as a function of total phenolics extract.

The total phenolic content of actinomycetes samples is depicted. Phenolic compounds in the extracts have redox properties, allowing them to act as antioxidants. The high antioxidant activity showed by the section has a positive relationship with TPC activity. Previous studies have shown that the capacity of the antioxidant is highly associated with the total flavonoid content and total phenolic compounds. The higher phenolic content in the extract is responsible for bioactivity; therefore, the extract is expected to exhibit good antioxidant and antibacterial activities. ACT14 showed good activity because of the higher phenolic content in it.

3.6.4 Total Antioxidant Activity (PhosphomolybdenumAssay)

The total antioxidant activity of the actinomycete extracts were measured using spectrophotometry and the phosphomolybdenum method, which is based on the

reduction of Mo (VI) with petroleum ether and subsequent formation of a phosphate/Mo (V) complex at an acidic pH, absorbance at 695nm[38]. The biologically active compounds extracted from the fractions showed very high antioxidant activity overall. Figure 9 depicts the results of the experimental sample and the equivalent of the standard antioxidant (ascorbic acid). The total antioxidant capacity of the extracted samples were 0.062 ± 0.004, 0.052 ± 0.003, 0.062 ± 0.001, 0.060 ± 0.005 and 0.048 ± 0.001 AA/g, respectively (ACT-10, ACT-11, ACT-13, ACT-14, and ACT-15), while the standard antioxidant ascorbic acid showed 0.074±0.002AA/g antioxidant activity.

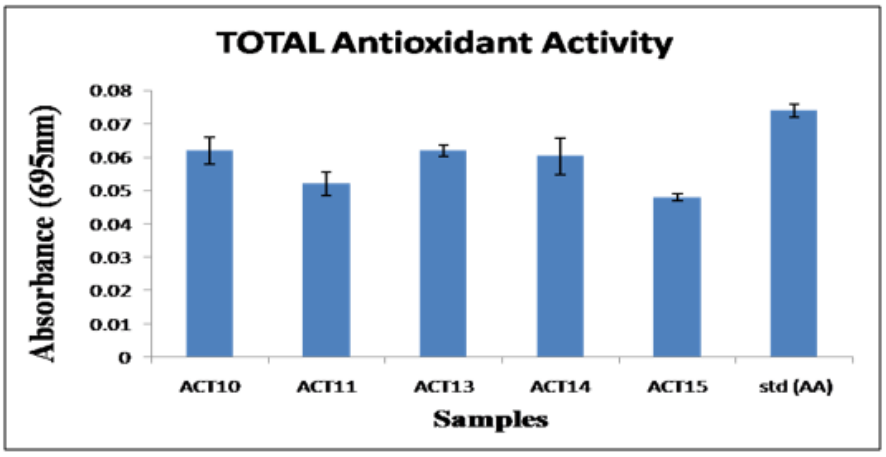

Fig 9: Free radical scavenging activities of five extracts of actinomycetes and ascorbic acid

The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the extract possessing antioxidant compounds. All samples showed good antioxidant activity when compared with the standard ascorbic acid. Therefore, it is suggested that the presence of effective antioxidants in all the fractions.

3.6.5 Total Reducing Power

The five fractions' reducing power was determined by reducing Fe^{3}+ to Fe^{2}+ in the presence of ascorbic acid. As the concentration of the extract increases, the absorbance of the reaction mixture at 700 nm increases, indicating that the potential of the extract reduces. The measured absorbance values ranged from 0.86 to 1.06. Among the tested extracts, the ACT10 extract showed the highest reducing power, although the reducing activity was lower than that of standard ascorbic acid, as shown in Figure 10. The reduced ability of the test fractions decreases in the following order: the extraction fraction ACT-14, ACT-15, ACT-11and extract ACT-13, respectively. The reducing activity of actinomycete extract is an important indicator of its potential antioxidant activity, the reducing ability of extracted samples were 1.059 ± 0.01, 0.748 ± 0.008, 0.395 ± 0.07, 0.959 ± 0.01 and 0.862 ± 0.059 AA/g, (ACT-10, ACT-11, ACT-13, ACT-14 and ACT-15). The antioxidant activity of standard ascorbic acid was 1.18 ± 0.03 µg /ml.

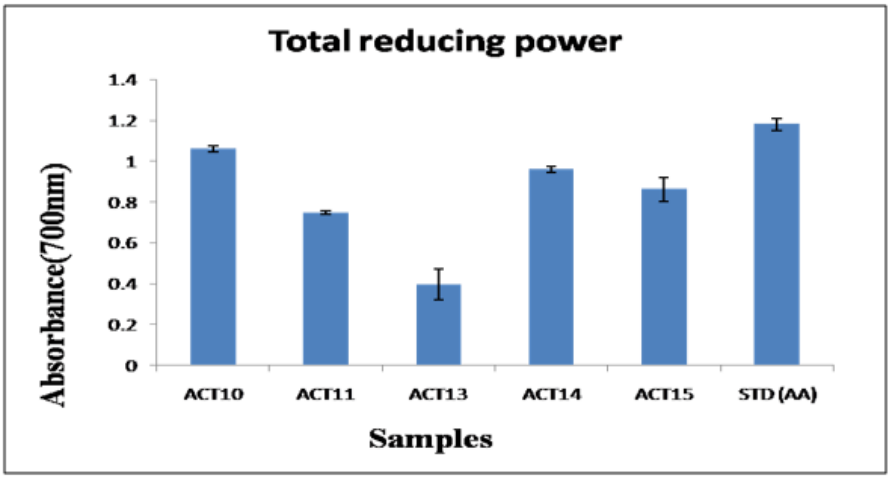

Fig 10: Ferric-reducing activities of five extracts of actinomycetes and ascorbic acid.

In this assay, the presence of e^{-} -donating compounds resulted in reduced Fe^{3+} (ferricyanide) into Fe^{2+} (ferrous). In addition, the reducing potential of the extracts was measured, showed a general increase in activity when the concentration was increased. Among the tested extracts, the extract of ACT10 and ACT14 possessed the highest reducing capacity of free radicals scavenging with an absorbance of 700nm.

3.7 Molecular Identification of Actinomycetes

The genomic DNA of the isolate was isolated using a DNA purification kit (Himedia). The DNA was confirmed by staining with ethidium bromide in 1% agarose. The PCR product of culture (ACT-14) was analyzed by agarose gel electrophoresis (1%), and the size (744bp) was determined and sequenced. The

NCBI BLAST tool was used to identify similar sequences. The 16S rRNA of the culture (ACT14) and was deposited in NCBI GenBank. Our results showed that the selected isolate ACT14 was a novel actinomycete that showed 85% similarity with *Pseudonocardia Antarctica*. The Gen Bank accession number of the submitted 16S rRNA sequence of ACT14 is MK973004.1.

3.7.1 Phylogenetic analysis of 16S rRNA sequence

The 16S rRNA gene of ACT14 was amplified and sequenced (MK973004.1). The partial 16S rRNA gene sequence of ACT-14 covered a stretch of 744bp, with an average GC content of 56.18%. The BLASTn analysis of nucleotides (Table 3) was similar to *Pseudonocardia sp.* S76 (JX007993.1) was 85%. The nucleotide sequences of the type strain were retrieved from the NCBI, and the phylogeny was studied (Figure 11). The phylogenetic position of this strain is in a cluster that contains *Pseudonocardia sp.* HH130629-09 (CP011868.1), *Pseudonocardia sp.* Ae20 16S ribosomal (FJ490530.1), and *Pseudonocardia sp.* YIM M10931-1 (JQ653115.1). *Pseudonocardia sp.*ACT-14 is placed on a single branch and shared with a closed group, with 99% query coverage and 85% sequence identity.

Table -3: The BLASTn results of 16S rRNA (ACT14) according to the NCBI database

Description	Accession number	Maximum query cover	Maximum score	Total score	Maximum identity (%)
Pseudonocardiasp S76 16S ribosomal RNA gene, Partial sequence	JX007993.1	100%	734	734	85%
Pseudonocardia sp. Ae20 16S ribosomal RNA gene, Partial sequence	FJ490530.1	99%	715	715	85%
Pseudonocardia sp. HH130629-09, complete genome	CP011868.1	100%	712	2136	84%
Pseudonocardia sp. YIM M10931-1,16S ribosomal RNA gene, partial sequence	JQ653115.1	84%	712	712	84%
Pseudonocardiaantarctica strain 13675 16S ribosomal RNA gene, partial sequence	JN180177.1	100%	712	712	84%
Pseudonocardia sp. MVT7 16S ribosomal RNA gene, partial sequence	EU931094.1	100%	712	712	84%
Pseudonocardia sp. CASMBPK11,16S ribosomal RNA gene, partial sequence	JX502844.1	97%	710	710	85%
Streptomyces aurantiogriseus strain 15747 16S ribosomal RNA gene, partial sequence	JN180195.1	99%	710	710	84%
Streptomyces aurantiogriseus strain NBRC 12842 16S ribosomal RNA gene, sequence partial sequence	JF950274.1	99%	710	710	84%

Table 3 depicts the BLASTn results of 16S rRNA (ACT14) according to the NCBI database. The nucleotide sequence or FASTA sequence of ACT14 (744bp) was selected for the BLASTn and performed, The BLASTn analysis of nucleotides showed that the similarity with *Pseudonocardia sp.* S76 (JX007993.1) was 85%. The phylogenetic position of this strain is in a cluster that contains *Pseudonocardia sp.* HH130629-09 (CP011868.1), *Pseudonocardia sp.* Ae20 16S ribosomal (FJ490530.1), and *Pseudonocardia sp.* YIM M10931-1 (JQ653115.1). *Pseudonocardia sp.*ACT-14 isplacedona a single branch and shared with a closed group, with 99% query coverage and 85% sequence identity.

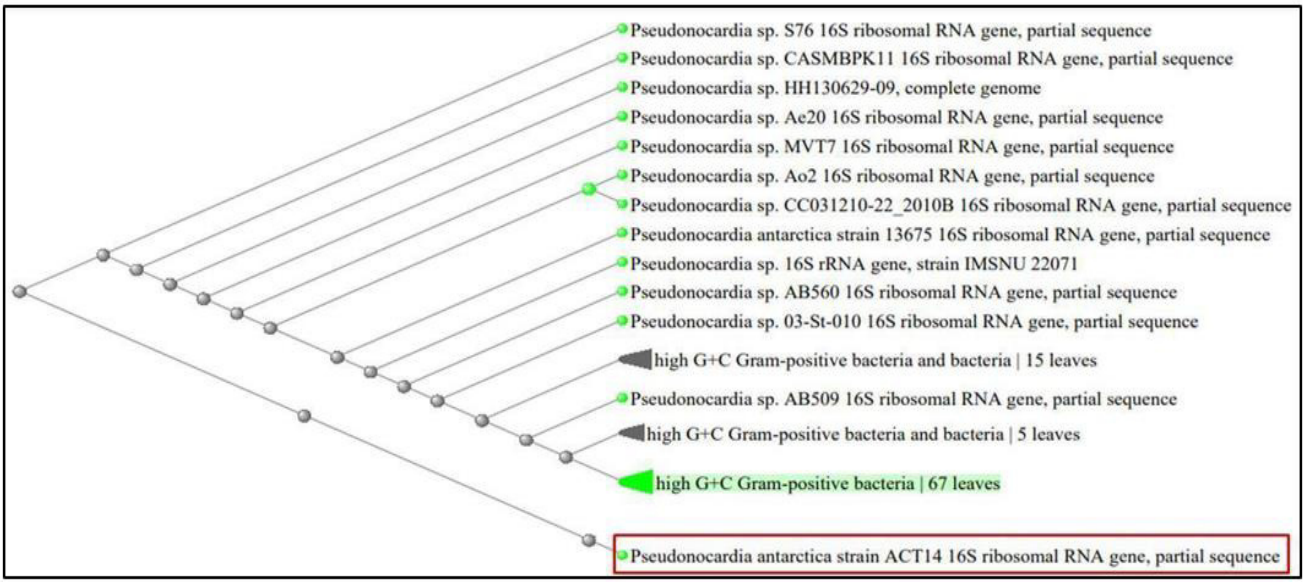

Fig 11: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain ACT-14 and closely related members of the genus *Pseudonocardia* as a reference strain

Figure 11 depicts the phylogenetic analysis of ACT14, sequences selected based on maximum identity score. All sequences are aligned using the MUSCLE 3.7 program. Next, Gblocks 0.91b was used to cure the resulting linear sequence. These Gblocks eliminate divergent regions (alignment noise suppression) and poor positions. Finally, the aLRT program PhyML 3.0 was used for phylogenetic analysis, with HKY85 as a representative model. Finally, for rendering trees, the Tree Dyn 198.3 program was used.

4. DISCUSSION

The current work analyzes the antibacterial activity, antioxidant activity, and molecular phylogeny of rare actinomycetes isolated from Sambhar Salt Lake, India. Physicochemical analyses reveal that this environment has significant salinity and that numerous halophilic actinomycetes were produced previously. Similar high saline conditions have been investigated several times as a source of biotechnologically important secondary metabolites from actinomycetes[39, 40].Marine bacteria have developed unique metabolic and physiological abilities to live in extreme conditions, allowing them to create compounds that terrestrial bacteria cannot produce. Actinobacteria are widely known to produce secondary metabolites, essential in preventing multi-drug-resistant infections. Actinobacteria develop spores that are often resistant to desiccation and exhibit some great extent of sensitivity to environmental fluctuations to adapt to harsh conditions compared to other microbes[41]. As a result, revealing the molecular phylogeny of actinomycetes that live in such hypersaline habitats is extremely important. The selective isolation methods used in these studies with diverse media and serial dilution methods to acquire actinomycetes successfully isolated rare actinomycetes *Pseudonocardia* (non-*Streptomyces*). Similarly, taxonomically diverse actinomycetes have been isolated from saline environments using techniques for selective sample processing and isolation medium[42].In the present study, Soil samples were taken from the top layer at depths of 5-10cm. The topsoil, which contains the majority of biological activity, is known to be rich in inorganic materials and bacteria[43], the selective isolation methods used a range of physical and chemical pretreatment techniques to selectively separate actinomycetes and were successful in isolating both *Streptomyces* (a common actinomycete) and non-*Streptomycetes* such as *Pseudonocardia*[44.45]For primary screening, the cross streak, and agar plug methods have utilized a total of 5 different isolates were screened, one promising *Actinobacteria* strain, identified as *Pseudonocardia Antarctica* ACT14 showed the novelty with antagonistic properties. The phylogenetic position of the *Pseudonocardia Antarctica* ACT14 suggested that isolated strains from Sambhar Lake have a potentially diverse arrangement with novelty which can be useful for many of the applications and can be explored broadly. The culture medium, SCA, was the best for isolating marine *Pseudonocardia Antarctica* ACT14. The strain showed optimum growth at 30°C on SCA media. *Pseudonocardia Antarctica* ACT14 was characterized morphologically and microscopically[46, 47].Using the well-known eubacterial phylogenetic marker, 16S rRNA gene, the isolated actinomycete phylogenetic relationship was studied[48]. One was chosen as a representative strain in this investigation from the total actinomycete isolates. A phylogenetic study of representative isolate ACT14 16S rRNA gene sequences showed that the acquired actinomycete is *Pseudonocardia Antarctica*.The16S rRNA gene sequencing and phylogenetic analysis revealed that ACT14 is a novel strain, having an identity below 85% as shown in figure 11. Phylogenetic analysis showed that the ACT14 strain was closely related to novel *Actinobacteria* bacteria such as *Pseudonocardiasp* S76 (JX007993.1) and *Pseudonocardia sp.* Ae20 (FJ490530.1), *Pseudonocardia sp*. HH130629-09 (CP011868.1), *Pseudonocardia sp*. YIM M10931-1 (JQ653115.1), isolated from different geographical locations, including deep-sea sediment[49].*Streptomyces* was found to be the most prevalent genus among actinomycetes isolated from inland solar salterns. *Pseudonocardia* members have been discovered in hypersaline marine habitats [50, 51]. In the current study, isolates linked with *Pseudonocardia* were obtained from an inland hypersaline environment. The presence of members associated with *Pseudonocardia*, previously discovered in inland solar salterns, is linked to the current study. The phylogenetic trees of the 16S rRNA isolate show that it may represent a new species. To characterize the new species, more polyphasic taxonomic research is required. The methods now used for bacterial systematics include complete 16S rRNA gene sequencing and phylogenetic tree comparisons, DNA-DNA hybridization studies with related species, analyses of molecular markers and signature pattern(s); the 'polyphasic approach' for bacterial systematics refers to the combination of genotypic, chemotaxonomic, and phenotypic approaches for establishing microbe taxonomic position[52].This polyphasic taxonomy does not depend on a theory, hypothesis, or set of principles, instead giving a pragmatic approach to a consensus form of taxonomy that incorporates all available facts to the greatest extent possible. Polyphasic taxonomy will have to deal with (i) massive volumes of data, (ii) high numbers of strains, and (iii) data fusion (data aggregation), which will necessitate effective and centralized data storage in the future[53]. Antimicrobial activity against various bacteria was tested to assess the promise for antibiotic development programs of saltern-based actinomycetes. Rare actinomycetes were found to have antimicrobial action. *Pseudonocardia,* a rare genus, was discovered to be more potent against tested bacteria. The significant inhibitory effects of this rare actinomycete with previously studied biotechnological relevance of rare actinomycetes[40, 54] concluded that these actinomycetes might be useful in producing antibacterial drugs. Some of the strains are presently being investigated to determine the nature of antibacterial compounds and their distinctiveness. The crude extract was examined and shown to have strong antioxidant activities, which can be valuable for future research development to make it industrially useful. The radical scavenging activity was concentration-dependent and increasing the concentration gradually enhanced the activity[55]. The DPPH free radical scavenging test was widely utilized to assess antioxidant activity. Antioxidants react with DPPH, reducing the quantity of DPPH molecules by the number of freely available hydroxyl groups[56]. The degree of DPPH scavenging activity is determined by its capacity to transfer hydrogen proton.[57]Accordingly at 10mg/mL concentration, ethyl acetate extract of *Streptomyces species* VITSTK7 isolated from the marine environment of the Bay of Bengal displayed 43.2% DPPH scavenging activity and 51% metal chelating activity. [58] Similarly, the antioxidant activity of three marine actinobacteria isolated from Nicobar Island marine sediments, whereas phenolic compounds recovered from *Streptomyces sp.* LK-3 displayed 76% DPPH scavenging activity at 100 µg/mL. To summarize, our research on new actinomycetes from coastline[59] and inland (this study) revealed the presence of actinobacteria in high-salinity Indian solar salterns. This research contributes to our understanding of

solar saltern-associated actinomycetes and enhances the number of actinomycetes available for drug development and antioxidant activities.

5. CONCLUSION

Sambhar Lake is a complex reservoir with diverse microorganisms that can survive tremendous pressure, salinity, and temperature variations. Microorganisms survive in harsh environments and develop physiological and metabolic abilities that allow them to synthesize metabolites that other terrestrial organisms cannot produce. In this study, actinomycete ACT14 (*Pseudonocardia Antarctica*) was isolated from the Sambhar Lake and showed novelty with antagonistic properties. Primary antimicrobial analyses helped in the selection of potent strains for further studies. Secondary screening helped in quantifying the activity of various culture extracts. Some of the bioactive compounds were as effective as well-known antibiotics and showed broad-spectrum activities. Bioactive compounds were extracted using different solvents; antioxidant activities were performed for various assays; However, ACT10 and ACT14 were more active than the other isolates. The extracted biologically active compounds with significant antibacterial and antioxidant activity have been used in further research, and efforts are now being made to remove biologically active compounds in large amounts for further characterization.

6. ACKNOWLEDGEMENT

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7. AUTHORS CONTRIBUTION STATEMENT

The authors confirm the contribution to the paper as follows: The Study conception and design was supervised by Dr. Divya Shrivastava and Dr. Indrani Jadhav. Mr. Vishal Mathur carried out the research work and Dr. Kapilesh Jadhav and Mr. Vishal Mathur wrote the manuscript, interpreted the results and verified the final manuscript. All authors read and agreed the final manuscript.

8. DATA AVAILABILITY

The manuscript includes all datasets generated or analyzed during this study.

9. CONFLICTS OF INTEREST

Conflict of interest declared none.

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