Isolation and characterization of marine actinomycetes – bioprospecting for antimicrobial, antioxidant and anticancer properties

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Abstract

The novel therapeutic agents are frequently produced from marine actinomycetes and are still being explored due to their vast diversity. In this research, we isolated five marine actinomycetes (BN01, BN12, BN13, BN14 and GB15) from the soil sediments of the coastal region of Chennai, Tamil Nadu, India. The morphological and physicochemical properties of the isolates were characterized. The 16S rRNA sequencing analysis showed that the isolates were belonging to two genera namely *Streptomyces* and *Saccharopolyspora*. The extracellular crude secondary metabolites were extracted from the culture filtrate of all the marine actinomycetes isolated and tested for the antimicrobial, antioxidant and anticancer potential adopting standard methods. The results revealed that the extracts of the isolates BN01, BN14, and GB15 exhibited antimicrobial activity against *M. luteus*, *E. coli*, and *S. aureus,* with the maximum zone of inhibition of 19 mm, 21 mm, and 27 mm respectively, at the concentration of 10 mg/mL. The extract of the isolate BN12 showed excellent radical scavenging activity of 97.75 % at the concentration of 60 µg/mL and has the half-maximal inhibitory concentration (IC_{50}) of 8.66 µg/mL. The anticancer activity results showed that the extract of the isolate BN12 had better inhibitory activity against the MCF 7 cell line with the IC_{50} value of 261.01 µg/µL.

Introduction

The marine environment is an excellent source of unique plants, microbes, algae, and animals for the production of biologically active materials (Almaary et al. 2021). Recent research is focusing on the marine environment for the discovery of new biologically important materials compared with the terrestrial environment (Leetanasaksakul and Thamchaipenet 2018). The marine microorganisms can grow at extreme temperatures, pressure, and salinity, and hence they can produce novel bioactive compounds (Abdelmohsen et al. 2014). Bacteria are the most dominant group of marine microorganisms which are easy to isolate and characterize. More new compounds with biological properties have been produced from marine bacteria.

Of marine microorganisms, actinomycetes are known as the greatest source of antibiotics with different chemical structures. The actinomycetes come under the phylum "*Actinobacteria*" and are mostly Gram-positive bacteria typically characterized by substrate and aerial mycelia on different solid media and form spores with different surface morphology (Nathan and Kannan 2020). The colonies often grow as extensive mycelia, like a fungus. Actinomycetes species are commonly found in terrestrial soils, plants, and fresh water, and are also found in marine sediment, water, and sponges. Only 28 genera with 83 species of actinomycetes have been identified(Selim et al. 2021). Recently, it has been reported that secondary metabolites from marine microorganisms have potential bioactive compounds showing anti-bacterial, anti-cancer, anti-fungal, anti-viral, anti-parasitic, anti-malarial and antifouling properties (Siddharth and Rai 2019).

The secondary metabolites are naturally produced by plants and microorganisms as a metabolic by-product, and they have been used as excellent lead compounds that can be modified structurally to enhance their drug efficiency (Al-Ansari et al. 2020). Among 200,000–250,000 metabolites with bioactivity, 50,000 bioactive compounds are produced by actinomycetes fermentation and Streptomyces species alone produced 35,000 compounds (Rashad et al. 2015; Rajivgandhi et al. 2018). Some of the secondary metabolites produced by the actinomycetes are enzyme inhibitors, antibiotics, antivirals, antitumor compounds, vitamins and pesticides (Rashad et al. 2015). Lavanducyanin and Naphthomevalin are bioactive compounds produced from Streptomyces sp. CPCC 203577 acts against gram-positive bacteria, Shellmycin A-D produced from Streptomyces sp. Shell-016 and Litoralimycins A and B from *Streptomonospora sp.* M2 has anticancer activity (Goel et al. 2021). Several reports have revealed that the secondary metabolites derived from marine actinomycetes have an enormous range of activities like antiparasitic, antiprotozoal, antitumor, antimicrobial, and antifouling activity (Kamjam et al. 2017). For example, the crude extracts of endophytic actinomycetes from marine sea grass exhibited excellent antibacterial activity against *K. pneumoniae* (Almaary et al. 2021).

The actinomycetes isolated from the Lagos lagoon exhibited anticancer potential against the HeLa cell line (Davies-Bolorunduro et al. 2019). The marine-derived Streptomyces species produced bicyclic peptides which displayed antifungal and cytotoxic activity against P388 murine leukemia cells (Karim et al. 2021). The crude extracts of *Streptomyces canus* from Mongolian soils showed antiprotozoal activities against *Toxoplasma* and other malarial parasites (Pagmadulam et al. 2020). This study aimed to evaluate the antimicrobial, antioxidant and cytotoxic activity of the marine actinomycetes.

Materials And Methods

Sample collection, processing and isolation of actinomycetes

The soil sediment samples were collected from the coastal regions of the Bay of Bengal near Chennai, Tamil Nadu, India, at three different locations (13°00'N, 80°27'E; 12°91'N, 80°25'E; 12°79'N, 80°25'E) and dried for a week in a sterile condition (Makkar and Cross 1982). The completely dried sediment samples were diluted serially, and plated on CSPY-ME medium (Casein- 3g, Maize starch- 10g, Peptone- 1g, Yeast extract- 1g, Malt extract- 10g, K_2HPO_4- 0.5g, Sea water- 1000mL, pH- 7.5, Agar- 20g) prepared in aged sterile seawater. The medium was amended with fluconazole (50 µg/mL) to inhibit the fungal growth. The agar plates were incubated at room temperature for 7–14 days for the growth of actinomycetes (Biji 2003).

Morphological, physiological and biochemical characterization

Microscopic Analysis

The coverslip culture method was used to determine the spore and mycelial morphology of the marine actinomycetes isolates. Briefly, the isolates were streaked on a Starch casein agar plate (Starch- 10g, Vitamin free casein- 0.3g, $CaCO_3$- 0.02g, $Fe_2SO_4.7H_2O$- 0.01g, $MgSO_4 \cdot 7H_2O$- 0.05g, KNO_3- 2g, K_2HPO_4- 2g, NaCl- 2g, Sea water- 1000mL, Agar- 20g, pH- 7.2 ± 0.2) and a sterile coverslip was inserted into the streaked plates at 45° angle and incubated at 28 ± 2°C for a week. After the growth of mycelia, the coverslip was taken out from the agar plate and observed under the light microscope for the spore and hyphal morphology, structure, and arrangement of the spore chain on aerial mycelium and number of spores (Shirling and Gottlieb 1966a).

Colour Determination and pigment production

The colour of aerial and substrate mycelium of the actinomycetes was observed in four different media, namely M2, M3, M4, and M5. [M2- Yeast Extract-Malt Extract agar; (Yeast extract- 4g, Malt extract- 10g, Dextrose- 4g, Sea water- 1000mL, pH- 7.3, Agar- 20g); Trace salts solution (For the preparation of M3, M4, M5 and M7) $FeSO_4.7H_2O$- 0.1g, $MnCl_2 \cdot 4H_2O$- 0.1g, $ZnSO_4 \cdot 7H_2O$- 0.1g, Distilled H_2O- 100mL;M3- Oatmeal agar (Oatmeal- 20g, Sea water- 1000mL, Agar- 18g, Trace salts- 1mL); M4- Inorganic salts-Starch agar (Solution-I Starch- 10g, Sea water- 500 mL; Solution-II K_2HPO_4 (anhydrous)- 1g, $MgSO_4 \cdot 7H_2O$- 1g, NaCl- 1g, $(NH_4)_2SO_4$- 2g, $CaCO_3$- 2g, Trace salts solution- 1 mL; Sea water- 500 mL, pH- 7- 7.4, Agar- 20g); The solution-I (starch suspension) and the solution-II (salts solution) were mixed and autoclaved at 121°C for 15–20 min; M5-Glycerol-asparagine agar (L-Asparagine- 1g, Glycerol- 10g, Anhydrous K_2HPO_4 – 1g, Trace salts solution- 1 mL, Sea water- 1000mL, Agar- 20g, pH- 7.0-7.4). The isolated cultures BN01, BN12, BN13, BN14, and GB15 were streaked onto the Petriplates and incubated for 2 weeks at room temperature. The colour of aerial mycelium and substrate mycelium was observed on 7 and 14 days. The Tresner-Backus colour series (Red, Yellow, Green, Blue, Violet, Gray, or White) was used to determine the colour of the aerial and the substrate mycelium. The production of soluble pigments and melanin pigment was observed by using Peptone-Yeast Extract Iron agar (Peptone iron agar (dehydrated)- 36g, Yeast extract- 1g, Sea water- 1000mL, pH- 7- 7.2) and Tyrosine agar medium (Glycerol- 15mL, L-tyrosine- 0.5g, L-asparagine- 1g, Anhydrous K_2HPO_4 – 0.5g, $MgSO_4 \cdot 7H_2O$- 0.5g, NaCl- 0.5g, $FeSO_4 \cdot 7H_2O$- 0.01g, Trace salts solution- 1 mL, pH- 7.2–7.4, Sea water- 1000mL ,Agar- 20g)(Shirling and Gottlieb 1966a).

NaCl Tolerance

The NaCl tolerance of the actinomycetes was observed by growing in varying concentrations of NaCl (0, 2, 4, 6, 8, and 10%) amended Starch-Casein agar medium. The loop of actinomycetes isolates was streaked on the agar plates and incubated at 28 ± 2°C. The growth on different concentrations of NaCl was observed after 7 and 14 days (Williams et al. 1983).

Organic compound degradation

The ability of actinomycetes to degrade organic compounds was determined by using Bennet's agar medium (Yeast extract- 1g, Beef extract – 1g, Tryptone- 2g, Dextrose- 10g, Sea water- 1000mL, Agar- 15g, pH- 7.3 ± 0.2) amended with organic compounds such as Xanthine (0.4%, w/v), Hypoxanthine (0.4%, w/v), Adenine (0.5%, w/v), Tyrosine (0.5%, w/v), and Casein (1%, w/v). The prepared agar plates were streaked with the actinomycetes isolates and incubated for 7–14 days at room temperature. The clear zone formation around the colonies determines the degradation of organic compounds (Sarika et al. 2021).

Carbon Utilization

The ability of the marine actinomycetes to utilize various carbon sources such as lactose, maltose, arabinose, sucrose, -fructose, l-inositol, galactose, rhamnose, and raffinose was observed in basal mineral salts agar medium ($(NH_4)_2SO_4$ – 2.64g, KH_2PO_4 – 2.38g, $K_2HPO_4.3H_2O$ – 5.65g, $MgSO_4.7H_2O$- 1g, Pridham and Gottlieb trace salts- 1.0 mL, Sea water- 1000mL, pH- 6.8–7, Agar- 15g) with 1% of carbon sources. (Pridham and Gottlieb trace salts (g/L) $CuSO_4 \cdot 5H_2O$- 6.4g, $FeSO_4 \cdot 7H_2O$- 1.1g, $MnCl_2 \cdot 4H_2O$- 7.9g, $ZnSO_4 \cdot 7H_2O$- 1.5g**).** A loopful of cultures streaked onto the agar plates and incubated at room temperature for 7–14 days. The positive control is D-glucose and the negative control is without a carbon source (Sarika et al. 2021).

Enzyme Production

The production of industrially vital enzymes like amylase, cellulase and protease was observed in Nutrient agar medium (Peptone- 5g, Beef extract- 3g, NaCl- 5g, Sea water- 1000mL, Agar- 20g, pH- 7.0 ± 0.2) containing starch (0.5%), carboxymethylcellulose (0.5%), and gelatin (0.5%) respectively. A clear hydrolytic zone around the colonies after the addition of indicator solutions, Gram's iodine, 0.3% Congo red, and saturated solution of ammonium sulphate in 1N HCl, respectively, were considered as positive. The nitrate reduction was determined by adding 0.2 ml of nitrate reagent A (Sulfanilic acid- 0.8g, 5N Acetic acid- 100mL) and reagent B (α-Naphthylamine- 0.5g, 5 N Acetic acid- 100mL) to the cultures in nitrate broth (Peptone- 5g, Beef extract- 3g, KNO_3- 1g, NaCl- 30g, Sea water- 1000mL pH- 7.0 ± 0.2). The formation of pinkish-red colour after the addition of reagents was positive. A few drops of twenty volumes of hydrogen peroxide were added to seven day old colonies of selected isolates grown on Bennett's agar medium, which resulted in the evolution of oxygen with air bubble formation on the surface of the colonies determined as catalase positive. Oxidase production was determined by the addition of broth culture on the oxidase disc (HiMedia, India). The formation of blue colour within 30 seconds was scored as positive (Williams et al. 1983).

Molecular Characterization

Isolation of genomic DNA

The non-enzymatic method was used to isolate the genomic DNA from the actinomycetes isolates (Sharma and Singh 2005). The isolates were grown on M-2 broth and 2mL of each culture broth were taken into 2mL Eppendorf tubes. It was then centrifuged at 5000 rpm for 5 mins at room temperature. The cell pellets were collected and suspended in 200 µL of TE buffer (Tris-HCl – 10 mM, EDTA – 1 mM, pH 8.0), 50ng of RNase and 400 µL of solution I (Sarkosyl – 1%, NaCl – 0.5M, SDS – 1%). The tubes were mixed well, incubated at 37°C for 10 min and shook intermittently for every five minutes. Then, PCI mixtures (Phenol : Chloroform : Isoamyl alcohol – 25 : 24 : 1) of equal volume was added immediately and mixed by inverting the tubes. The tubes were again centrifuged at 10,000 rpm for five minutes at 4°C. The supernatants were collected into another Eppendorf tubes to which isopropanol (0.6 volume) and 3M sodium acetate (0.1 volume) at pH 5.2 were added and tubes were inverted 4–6 times gently for mixing. Then, the tubes were centrifuged at 10,000 rpm for five minutes at 4°C and pellets were collected by discarding the supernatant. The collected pellets were washed by adding 70% ethanol of 1mL and allowed for centrifugation at 10,000 rpm for five minutes at 4°C. After discarding supernatant, the pellets were air dried and re-suspended in 100 µL TE buffer. The isolated DNA was run on agarose gel electrophoresis (0.8%) for confirmation.

PCR amplification and 16S rRNA sequencing

The 16S rRNA gene of the marine actinomycetes isolates was amplified from genomic DNA. Universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT – 3') were used to amplify 16S rRNA gene (Weisburg et al. 1991). The PCR reaction was performed in a final volume of 25 µL, which was composed of template DNA (1µL), 20 pM of each primer, 2X Master Mix (12.5 µL) containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs and 2.5 U of *Taq* DNA polymerase with the appropriate reaction buffer under the following condition: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 5 min and finally held at 4°C. The amplified product was examined with agarose gel electrophoresis and sequenced. The 16S rRNA gene was purified and sequencing was carried out on Illumina MiSeq platform at Eurofins Genomics India Pvt.Ltd.

Genbank submission

The 16S rRNA gene sequence of the marine actinomycetes isolates BN01, BN13, BN14 and GB15 was identified by comparing similarity with the reference sequences contained in EMBL/GenBank/DDBJ database banks, using the NCBI Blast search available at http://www.ncbi.nlm.nih.gov and submitted at NCBI GenBank with the accession numbers.

Production of crude secondary metabolites

The production medium [M14; Glycerol- 70mL; Glucose- 30g; Beef extract- 30g; Peptone- 8g; $NaNO_3$- 2g; $MgSO_4.7H_2O$- 0.1g; $CaCO_3$- 3g; Distilled water- 1000mL pH- 7.0; K_2HPO_4- 2.5g; KH_2PO_4- 2.5g] was prepared for 250 mL. The seed culture was prepared by culturing actinomycetes isolates in the ISP-2 broth of each 50 ml for 3 days in an orbital shaker incubator at 28°C. Three days old seed culture was inoculated in the production media and incubated in an orbital shaker for 7 days at room temperature (Biji 2003).

Extraction of crude secondary metabolites

The production medium was harvested after 7 days and centrifuged at 7,000 rpm for 15 mins. The cell-free supernatant was subjected to a liquid-liquid extraction process using ethyl acetate as a solvent in a separating funnel (Gebreyohannes et al. 2013). After strong agitation, the upper layer of ethyl acetate was collected and condensed using a rotary evaporator. Further, the crude secondary metabolite was fractionated by using chloroform, ethyl acetate, and methanol.

Antimicrobial assay

The well diffusion method was used in determining the antibacterial and antifungal activities of the crude secondary metabolites. The Muller-Hinton agar (HiMedia, India) for bacterial pathogens *E. coli, P. aeruginosa, S. aureus, M. luteus* and potato dextrose agar (Potato- 200g, Dextrose- 20g, pH- 6.5, Distilled water- 1000mL Agar- 20g) for fungal pathogen *C. albicans* were prepared and the pathogens were swabbed onto the agar plates. The well was prepared by using a sterile cork-borer of 6 mm diameter and 50 µL of crude metabolites dissolved in DMSO (10mg/mL) was dispensed into the wells along with positive and negative control. The sample was allowed to diffuse for two hours and incubated at 37°C for 24h. The zone of inhibition was recorded for each well after 24h (Gebreyohannes et al. 2013). The positive control used for bacterial pathogens was tetracycline (50 µg) and for fungal pathogen was fluconazole (50 µg). The negative control used was dimethyl sulfoxide.

DPPH antioxidant assay

The antioxidant activities of the extracts were examined by DPPH radical scavenging assay (Rahman et al. 2015). DPPH (2,2-diphenyl-1-picrylhydrazyl) is a highly reactive free radical with a purple colour which can draw an electron from the donor. A DPPH methanol solution of 0.1mM was prepared and 1ml of DPPH solution was mixed with 1ml of extracts at varying concentrations of 20–100 µg/mL. The positive control was ascorbic acid (1 mg/mL). The reaction mixture was mixed and left for 30 min in dark conditions. The absorbance was measured at 517nm in a spectrophotometer. The decolourization of methanolic DPPH from purple to shades of yellow determines the radical scavenging activity of the crude extracts.

The percentage of scavenging activity was calculated using the following formula:

$$DPPHradicalscavengingactivity(\%) = \left[\frac{B0 - B1}{B0}\right] * 100$$

where, B0 is the absorbance of the standard and B1 is the absorbance of the sample. The percentage of free radical scavenging activity was blotted in a graph against the concentration of the sample, and IC_{50} values were calculated.

Anticancer activity

The MTT-based cytotoxicity activity of the *Streptomyces sp.* strain BN12 crude extract was tested against the human breast cancer cell line MCF-7 by a rapid colorimetric method (Mosmann 1983). The cells were seeded at the plating density of 10,000 cells/well in a 96-well plate containing a medium with 5% of Fetal Bovine Serum, and the plates were incubated for 2 days at 37°C and 5% CO_2 with 95% air and 100% relative humidity before the addition of crude extract. Then, the samples were added at different concentrations of 100–1000 µg/mL and again incubated for two days at 37°C with the same conditions. Then, 50 µL of MTT at the concentration of 5 mg/mL was added to each well and incubated for four hours at 37°C. The formazan crystals formed on MTT medium were solubilized in dimethyl sulfoxide (100 µL) and the absorbance was measured in a microplate reader at 570 nm.

The percentage cell inhibition was calculated using the following formula:

$$Cellinhibition(\%) = \left(100 - \frac{Absorbance(Sample) - Absorbance(Blank)}{Absorbance(Control) - Absorbance(Blank)}\right) * 100$$

2.8 GC-MS analysis

The GC-MS characterization of *Streptomyces sp.* BN12 crude extract was performed by Gas chromatograph and mass spectrometer GC-MS-5975 (AGILENT) of column DB 5 ms Agilent, film thickness of 0.25µm, dimension length of 30m, ID of 0.2mm. The temperature is between 70–300°C, 10°C/min with injection temperature at 240°C. The carrier gas used is helium with flowrate of 1.51 mL/min. The GC-MS analysis is equipped with GC-MS NIST-II library.

Results

Isolation of marine actinomycetes

The soil sediment samples collected from the coastal regions of Tamil Nadu, India were serially diluted and plated on a CSPY-ME medium. Fifteen colonies were selected based on resemblance to actinomycetes and sub-cultured in Starch-casein agar medium. In that, only 5 colonies were shown as actinomycetes based on clear, white and pale-yellow colonies with powdery spore formation and named as BN01, BN12, BN13, BN14, and GB15 (Fig. 1).

Note: Position for Fig 1

Morphological, physiological and biochemical characterization

Microscopic Analysis

The light microscopic analysis showed the status of mycelium, branched or unbranched, number of spores, and shape of spores. All the isolates formed very dense, extensively branched aerial and substrate mycelium. The spore morphology of the isolate BN01 showed coiled and the spore chain type was Spira (S). The isolates BN12 contained short chains of spores and the spore chain type was Retinaculum Apertum (RA). Meanwhile, the BN13 and BN14 exhibited a long chain of spores and Retinaculum Apertum (RA) type. The isolate GB15 formed straight short chain of spores (Fig 2).

Note: Position for Fig 2

Colour Determination and Pigment production

The morphological characterization shows that the colour of aerial and substrate mycelium of actinomycetes varies from white, grey, red, green, pink, orange, and brown. The isolate BN01 mycelium varies from yellowish grey, pinkish grey, brown, yellow, grey and yellowish brown whereas BN12 has greyish green, yellowish green, black, whitish grey and greyish yellow. The aerial and substrate mycelium of isolates BN13 and BN14 has colour varying from pinkish grey, yellowish grey, yellowish white, orange, greyish white and brown. The isolate GB15 mycelium has white, brown, yellow, orange and grey shades of colour. It was observed that the five isolates had not produced any soluble pigments or melanin (Table 1).

Note: Position for Table 1

NaCl Tolerance

The results show that the isolate BN01 has grown upto 8% NaCl concentration and better growth at 4% NaCl. The isolate BN12 had better growth at 2% NaCl and no growth at 6, 8, and 10% NaCl concentrations. The BN13 has moderate growth at 2, 4 and 6% NaCl concentrations whereas less growth at 8% and no growth at 10% NaCl. The BN14 has grown upto 8% of NaCl concentration and

better growth at 2, 4, and 6% of NaCl. The isolate GB15 had less growth at 2 and 4% of NaCl; moderate growth at 6 and 8%, and better growth at 10% of NaCl concentration (Table 2).

Organic compound degradation

The organic compound degradation showed that no isolates have degraded xanthine and adenine. Hypoxanthine and tyrosine were degraded by all the isolates except BN01, BN13, BN14 and GB15. Casein was degraded by BN01, BN12, BN14 and GB15 (Table 2).

Carbon utilization

It showed that BN01, BN13, BN14, and GB15 can strongly utilize glucose, fructose, lactose, and maltose since the growth of actinomycetes was good on the above carbon sources. The isolate BN12 moderately utilizes fructose. The galactose was moderately utilized by isolates BN01, BN12, and BN13 whereas highly utilized by BN14. The isolate BN15 did not utilize maltose and galactose since no growth was observed on the plates. All the isolates were found to moderately utilize sucrose and raffinose whereas the isolate BN15 did not utilize inositol and rhamnose. The utilization of arabinose was undetermined for isolates BN13 and GB15 (Table 2).

Enzyme production

All isolates have produced enzymes protease and amylase, and cellulase was produced only by isolates BN01, BN13, and BN14. This was confirmed by the formation of clear hydrolytic zones after the addition of indicator solutions. The oxidase enzyme was produced only by isolate BN14 and the production of catalase was observed in all isolates except BN13. The isolate BN12 and BN14 have highly produced catalase enzyme whereas the isolates BN01 and GB15 have moderately produced the catalase enzyme. The nitrate reduction was observed in isolates BN01 and BN12 (Table 2).

Note: Position for Table 2

Molecular Characterization

The NCBI blast analysis of 16S rRNA gene sequences showed that the actinomycetes isolates belong to the genera Streptomyces and Saccharopolyspora. The sequences were submitted into the NCBI GenBank along with the accession number namely Streptomyces rochei strain BN01 (ON042751), Streptomyces sp. strain BN13 (ON042452), Streptomyces sp. strain BN14 (ON042463) and Sacchropolyspora sp. strain GB15 (ON042476).

Based on the morphological, biochemical and molecular characterization, 4 isolates such as BN01, BN12, BN13 and BN14 belongs to the genera Streptomecetes and the isolate GB15 belongs to the genera Saccharopolyspora.

Antimicrobial assay

The crude secondary metabolites extracted from marine actinomycetes isolates were tested for antimicrobial activity. The results of the antimicrobial assay showed that the ethyl acetate fraction of Sacchropolyspora sp. strain GB15 exhibited a maximum zone of inhibition of 27±1.41 mm against the gram-positive bacteria S. aureus and 18±1.41 mm against E. coli at the concentration of 10 mg/mL. The ethyl acetate fraction of Streptomyces rochei strain BN01 showed a maximum zone of inhibition (19±1.41 mm) against M. luteus and 16.5±0.71 mm against E. coli. The methanol fraction of Streptomyces rochei strain BN01 isolates exhibited 19±1.41 mm and 18±1.41 mm inhibitory zones against S. aureus and E. coli respectively. The ethyl acetate fraction of Streptomyces sp. strain BN14 showed 210±1.41 mm of the zone of inhibition against E. coli meanwhile, methanol fraction showed 16.5±0.71mm of the zone of inhibition against E.coli. P. aeruginosa and C. albicans were moderately inhibited by fractions of all the marine actinomycetes isolates with the average zone of inhibition of 13 – 16 mm (Table 3). The results indicate that the crude

secondary metabolites of actinomycetes have a broad range of antibacterial activity against both gram-positive and gram-negative bacteria and antifungal activity.

Note: Position for Table 3

DPPH antioxidant assay

In this study, the EA fraction of Streptomyces sp. strain BN12 showed the highest radical scavenging activity of 97.75% at the concentration of 60µg/mL and has the half-maximal inhibitory concentration (IC_{50}) of 8.66 µg/mL. The ethyl acetate fractions of Streptomyces rochei strain BN01 and Saccharopolyspora sp. strain BN15 have increased radical scavenging activity of 89.81% and 77.59% at the concentration of 100 µg/mL respectively, and methanol fraction of Streptomyces rochei strain BN01, Streptomyces sp. strain BN12 and Saccharopolyspora sp. strain GB15 exhibited 69.70%, 88.77% and 87.56% of activity in the same concentration. The isolates Streptomyces sp. strain BN13 and Streptomyces sp. strain BN14 have moderate to low radical scavenging activity and the chloroform fractions of all the isolates do not have antioxidant properties (Fig. 3 & 4).

Note: Position for Fig 3

Note: Position for Fig 4

MTT-based cytotoxicity assay

The cytotoxic effect of the ethyl acetate fraction of Streptomyces sp. strain BN12 was evaluated against the human breast cancer cell line (MCF-7) and also compared with the Vero cell line. It has the IC_{50} value of 261.01µg/µL against the MCF-7 cell line and 563.3µg/mL against the Vero cell line. The morphological changes of the MCF-7 breast cancer cell line after the treatment with the ethyl acetate fraction of the isolate BN12 were evaluated under an inverted microscope (Fig. 5).

Note: Position for Fig 5

GC-MS analysis

The GC-MS analysis of the ethyl acetate crude extract of Streptomyces sp. strain BN12 was performed by comparing the mass spectra of the constituents with NIST library (Fig 6). Ten compounds were identified and listed the compounds in Table 4 with retention time, chemical formula, area covered, molecular weight and biological activity.

Note: Position for Fig 6

Note: Position for Table 4

Discussions

New pathogenic microorganisms and new diseases which are multidrug-resistant have been emerging and controlling such diseases with therapeutic measures has become a global concern. Actinomycetes have been targeted because of their ability to produce various new compounds to treat pathogenic and deadly diseases (Genilloud 2018). Different habitats have been explored for isolation of actinomycetes, and marine habitats are still being explored due to their vastness. In this research, three coastal regions of Chennai, Tamil Nadu, India have been explored and a total of 15 soil sediment samples were collected and the dried samples were used for the marine actinomycetes isolation. The five actinomycetes were isolated in CSPY-ME agar medium based on colony morphology, which were named as BN01, BN12, BN13, BN14, and GB15. All the actinomycetes isolates grew well on the starch casein agar medium. Similarly, Valan *et al*(2012) isolated 210 actinomycetes from marine sediment samples from the Andhra Pradesh coast of India and they have used starch casein agar medium for antibacterial and antifungal studies (Valan et al. 2012).

The isolated cultures were characterized by morphological and various physicochemical properties adopting standard methods (Shirling and Gottlieb 1966b). The spore morphology is considered as an important characteristic of the actinomycetes and in this study, the isolates BN13 and GB15 have oval-shaped short-chain spores. The colour of aerial and substrate mycelium, as well as the production of soluble pigment and melanin was observed in six different media, namely M2, M3, M4, M5, M6 and M7, and it revealed that the colour varied from white, grey, yellow, brown, red, pink, green, and black. Similar results were shown by (Ramachandran et al. 2019) as the mycelial colour varies from brown, yellowish brown, light grey, dark grey, and white. It also showed that the actinomycetes isolate had not produced any soluble pigments and melanin.

The isolate BN01 showed excellent growth at 4% NaCl, whereas BN12 showed at 2% NaCl. At concentrations 2, 4, and 6% NaCl, the isolates BN13 and BN14 have moderate and maximum growth tolerance, respectively. The isolate GB15 has good growth at 10% NaCl. These results were compared with (Valan et al. 2012) where all the marine actinomycetes isolated from sediment showed good growth at 2% NaCl, moderate growth at 4%, less growth at 6%, and no growth at 8% NaCl. All the isolates have degraded the organic compounds hypoxanthine and tyrosine except BN12, and except BN13, all isolates have degraded casein. But no isolates have degraded xanthine or adenine. Most of the isolated actinomycetes were dependent on glucose, fructose, lactose, galactose, maltose, sucrose, and raffinose as a carbon source. The isolate GB15 does not utilize maltose, galactose, inositol or rhamnose. Similar to this study, (Sarika et al. 2021) reported that the actinomycetes have utilized various carbon sources such as dextrose, arabinose, maltose, and lactose.

Since enzymes have been explored and used in the pharmaceutical industry, actinomycetes have been used in the production of enzymes. The amylase enzyme is used to hydrolyze starch and is applied in food, textile, paper and textile industries (Kafilzadeh and Dehdari 2015) whereas the protease enzyme is used in breaking down proteins into peptides and amino acids. The protease enzymes are produced by animals, plants, bacteria, and fungi. The enzymes protease and amylase were produced by all the isolates and it was confirmed by the formation of clear hydrolytic zones. This result was consistent with the report of (Chakraborty et al. 2021), where 61.42% of total isolates produced amylase and 42.85% produced protease. Cellulolytic bacteria have been used in the degradation of lignocellulosic biomass. The isolates BN01, BN13, and BN14 have produced cellulase by using the substrate carboxymethylcellulose and a similar result was reported by (Prasad et al. 2013), where the cellulose hydrolysis was found to be optimum by the isolates. Oxidase enzyme production was observed only in isolate BN14 and was confirmed by the formation of a dark blue colour in the oxidase disk after the addition of culture broth. The catalase enzyme was highly produced in isolates BN12 and BN14 whereas moderately produced in isolates BN01 and BN12. The isolates BN01 and BN12 have the ability to reduce nitrates, whereas others do not have nitrate-reducing capacity. It was confirmed by the formation of pinkish red colour after the addition of nitrate reagents to culture broth.

Based on the BLAST analysis, the isolate BN01 showed high similarity with *Streptomyces rochei* strain, the isolates BN12, BN13, and BN14 shared high similarity with *Streptomyces sp*. whereas the isolate GB15 displayed high similarity with *Saccharopolyspora sp*. Similarly, (Chakraborty et al. 2021) have characterized actinomycetes from marine sediments as *Streptomyces levis* strain KS46 by 16s rRNA molecular sequencing with 97.97% of sequence similarity.

The secondary metabolites were produced by initially growing the isolates in ISP-2 broth and transferred into the production medium. The crude secondary metabolites were extracted by the liquid-liquid extraction process by using ethyl acetate as a solvent. Further, crude secondary metabolites were fractioned using chloroform ethyl acetate and methanol. Antimicrobial, antioxidant, and anticancer assays were performed for the crude fractions. It shows that both gram positive and gram negative bacterial and fungal pathogens were susceptible to atleast one of the actinomycetes strain. The maximum zone of inhibition was exhibited by *Saccharopolyspora sp.* strain GB15. The gram-negative bacteria were found to be more resistant when compared with gram-positive bacteria due to the presence of lipopolysaccharide in gram-negative bacteria.. In contrast to this, (Rajivgandhi et al. 2019) showed methanol to be an excellent solvent for the antibacterial extract with a 29 mm zone of inhibition against *E. coli* followed by ethyl acetate and chloroform extracts.

The antioxidant radical scavenging activities of the extracts were analyzed by the change in colour from purple to yellow. It is observed that the ethyl acetate and methanol fractions of BN01 and BN12 have rapid colour changes from purple to yellow shades which show increased radical scavenging activity. But, in chloroform fractions, no colour changes were observed, and hence, they do not have radical scavenging activity. The ethyl acetate and methanol fractions of BN13, and chloroform and methanol fractions of BN14 show gradual colour changes from purple to yellow; hence they concluded to have medium radical scavenging activity,

whereas the methanol and ethyl acetate fractions of GB15 have rapid colour changes from purple to yellow, hence having high radical scavenging activity. since no colour change was observed in all the isolates. (Krishnamoorthy et al. 2020) reported that coral-reef associated actinomycetes *Saccharopolyspora* sp.IMA1 showed maximum radical scavenging activity of 65.3% at the concentration of 100 µg/mL. Similarly, 33% of radical scavenging activity at the concentration of 5 µg/mL was observed in *Streptomyces* sp. Al-Dhabi-100 isolated from marine environment reported by (Abdullah Al-Dhabi et al. 2020).

Since, the ethyl acetate fraction of *Streptomyces sp.* strain BN12 has excellent radical scavenging activity, the MTT assay was performed using the same fraction. The anticancer activity for the ethyl acetate fraction of *Streptomyces sp.* strain BN12 against human breast cancer cell line MCF-7 showed better cytotoxicity. The microscopic images showed that the treated MCF-7 cell line has less number of cells compared with the MCF-7 control. The inverted microscopic images show the changes in cell morphology of the MCF-7 cell line after treatment with the crude extract and a smaller number of cells observed compared with control. (Davies-Bolorunduro et al. 2019) reported the highest cytotoxicity against the AGS cell line by the crude extracts of *S. fulvissimus* isolated from the Lagos Lagoon sediment with the IC_{50} value of 0.030 mg/mL at the crude extract concentration of 0.01-5 mg/mL.

The GC-MS profiling showed presence of ten compounds in the ethyl acetate crude extract of *Streptomyces sp.* strain BN12 such as fatty acid esters, alcohols, flavonoids and ketones. Among 10 compounds, Isosativene, Flavone, Phytol, Morin, Octadecanoic acid 3-oxo- methyl ester and Oleic acid were found to have antioxidant, antibacterial, anti-inflammatory and anti-cancer activity (Kumar; Cushnie and Lamb 2005; Chen et al. 2013; Caselli et al. 2016; Park et al. 2016; Islam et al. 2018; Chenniappan et al. 2020; Kopustinskiene et al. 2020). Beekwilder et al reported that the compound 1-Butanone, 1-(2-hydroxyphenyl) have been used as food additive (Beekwilder et al. 2007). The compounds 2,4-Monoethylidene-l-xylitol, 2,6-Bis(1,1-dimethyl)-4-phenylmethylenecyclohexa-2,5-dien-1-one and 4,7-Methanoazulene decahydro-1,4,9,9-trtramethyl were not reported any biological activity. This result was compared with (Chakraborty et al. 2021), where 42 bioactive compounds were produced from ethyl acetate extract of *Streptomyces sp.* such as fatty acid esters, steroids, alcohols, fatty alcohols and ketone.

Conclusion

Actinomycetes are the most dominant group of microorganisms contributing to the production of a large number of secondary metabolites including antibiotics and other bioactive compounds. In this present study, a total of 15 samples were collected from coastal regions of Chennai, Tamil Nadu, India, and five actinomycetes were isolated based on the nature of colony morphology. The morphological, physiological, biochemical and molecular characterizations were performed to identify the isolated actinomycetes. Then crude secondary metabolites were extracted from actinomycetes to perform antimicrobial, antioxidant and MTT assays. The ethyl acetate fraction of the isolate *Saccharopolyspora sp.* strain GB15 exhibited excellent antibacterial activity against *S. aureus* (27 mm) followed by isolate *Streptomyces sp.* strain BN14 against *E. coli* (21 mm) at the concentration of 10 mg/mL. The ethyl acetate fraction of *Streptomyces sp.* strain BN12 exhibited excellent DPPH radical scavenging activity of 97.75% and IC_{50} value of 8.66 µg/mL at the concentration of 60 µg/mL. It also has better cytotoxicity against the human breast cancer cell line MCF-7 with the IC_{50} value of 261.01 µg/µL. The GC-MS profiling of ethyl acetate crude extract of *Streptomyces sp.* strain BN12 revealed 10 compounds with biological activity and some with no reports. The results shows that the marine actinomycetes are potential source of bioactive compounds.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Conceptualization: Mohanya Kumaravel, Kasinathan Kalimuthu; Methodology: Mohanya Kumaravel, Kasinathan Kalimuthu; Formal analysis and investigation: Kasinathan Kalimuthu; Writing – original draft preparation: Mohanya Kumaravel; Writing – review and editing: Kasinathan Kalimuthu, Dibyajyoti Haldar; Resources: Arumugam Perumal; Supervision: Kasinathan Kalimuthu, Dibyajyoti Haldar. Project administration: Kasinathan Kalimuthu.

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Tables

Table 1

Colour determination and pigment production

Isolate No.	Time	Media	Aerial Mycelium	Substrate Mycelium	Pigment	Melanin
BN01	14^{th} day	M2	Pinkish grey	Brown	-	-
		M3	Grey	Yellowish white	-	-
		M4	Yellowish grey	Yellow	-	-
		M5	Yellowish grey	Yellowish grey	-	-
		M6	Pinkish grey	Yellowish brown	-	-
		M7	Yellowish grey	Yellow	-	-
BN12	14^{th} day	M2	Greyish green	Yellowish brown	-	-
		M3	Greyish green	Greyish green	-	-
		M4	Greyish green	Greyish green	-	-
		M5	Greyish green	Yellowish green	-	-
		M6	Grey	Black	-	-
		M7	Whitish grey	Greyish yellow	-	-
BN13	14^{th} day	M2	Pinkish grey	Brown	-	-
		M3	Grey	Yellowish grey	-	-
		M4	Yellowish white	Yellowish white	-	-
		M5	Yellowish grey	Yellowish grey	-	-
		M6	Yellowish grey	Orange	-	-
		M7	Greyish white	Greyish yellow	-	-
BN14	14^{th} day	M2	Pinkish grey	Brown	-	-
		M3	Yellowish white	Yellowish white	-	-
		M4	Pinkish grey	Brown	-	-
		M5	Yellowish white	Yellowish white	-	-
		M6	Greyish yellow	Yellowish orange	-	-
		M7	Grey	Yellow	-	-
GB15	14^{th} day	M2	White, Green	Brown	-	-
		M3	White	White	-	-
		M4	Greyish white	Yellowish grey	-	-
		M5	Yellowish orange	Yellowish orange	-	-
		M6	White	Yellow	-	-
		M7	Yellowish white	Orange	-	-

Table 2

Biochemical and physiological characteristics of isolates

Characteristics	Isolates				
	BN01	BN12	BN13	BN14	GB15
NaCl Tolerance					
0%	++	++	+++	+++	+
2%	++	+++	++	+++	+
4%	+++	+	++	+++	+
6%	++	-	++	+++	++
8%	++	-	+	++	++
10%	+	-	-	-	+++
Organic degradation					
Xanthine	-	-	-	-	-
Hypoxanthine	+	-	+	+	+
Adenine	-	-	-	-	-
Tyrosine	+	-	+	+	+
Casein	+	+	-	+	+
Carbon utilization					
Glucose	++	++	++	++	++
Sucrose	+	+	+	+	+
Fructose	++	+	++	++	++
Lactose	++	++	++	++	++
Maltose	++	++	++	++	-
Galactose	+	+	+	++	-
Inositol	+	+	+	+	-
Arabinose	+	+	±	+	±
Rhamnose	+	+	+	+	-
Raffinose	+	+	+	+	+
Nitrate Reduction	++	++	-	-	-
Enzyme Production					
Protease	+	+	+	+	+
Cellulase	+	-	+	+	-
Amylase	+	+	+	+	+
Oxidase	-	-	-	++	-
Catalase	++	+++	-	+++	++

Note: + = positive; ++ = moderately positive; +++ = highly positive; - = negative; ± = undetermined

Table 3

Antimicrobial activity of the marine actinomycetes isolates

Samples	*Micrococcus Luteus*	*Pseudomonas Aeruginosa*	*Staphylococcus Aureus*	*Escherichia Coli*	*Candida Albicans*
***Streptomyces rochei* strain BN01**					
CHL	0	12.5 ± 0.71	11 ± 0	13 ± 0	12.5 ± 0.71
EA	19 ± 1.41	14 ± 1.41	15.5 ± 0.71	16.5 ± 0.71	0
MET	9.5 ± 0.71	0	19 ± 1.41	18 ± 1.41	0
POS	34 ± 1.41	15.5 ± 0.71	36.5 ± 0.71	30.5 ± 0.71	40.5 ± 0.71
***Streptomyces sp.* strain BN12**					
CHL	9.5 ± 0.71	13.5 ± 0.71	0	0	15 ± 1.41
EA	9.5 ± 0.71	12 ± 0	16 ± 1.41	0	10.5 ± 0.71
MET	0	13.5 ± 0.71	0	0	9.5 ± 0.71
POS	33 ± 1.41	31 ± 1.41	33.5 ± 0.71	29.5 ± 0.71	39 ± 0
***Streptomyces sp.* strain BN13**					
CHL	10.5 ± 0.71	10.5 ± 0.71	0	0	14.5 ± 0.71
EA	14 ± 0	15 ± 1.41	15.5 ± 0.71	12.5 ± 0.71	0
MET	16 ± 1.41	12.5 ± 0.71	0	0	0
POS	31.5 ± 0.71	18 ± 0	32 ± 0	28 ± 0	37.5 ± 0.71
***Streptomyces sp.* strain BN14**					
CHL	12.5 ± 0.71	9.5 ± 0.71	0	9.5 ± 0.71	15 ± 1.41
EA	15 ± 1.41	15 ± 1.41	16 ± 1.41	21 ± 1.41	13.5 ± 0.71
MET	0	0	16 ± 1.41	16.5 ± 0.71	15 ± 1.41
POS	29 ± 1.41	21.5 ± 0.71	32.5 ± 0.71	36 ± 1.41	37.5 ± 0.71
***Saccharopolyspora sp.* strain GB15**					
CHL	0	14 ± 1.41	13.5 ± 0.71	0	13.5 ± 0.71
EA	13.5 ± 0.71	12.5 ± 0.71	27 ± 1.41	18 ± 1.41	0
MET	0	10.5 ± 0.71	0	0	14.5 ± 0.71
POS	27.5 ± 0.71	24 ± 0	33.5 ± 0.71	32.5 ± 0.71	39.5 0.71

Table 4

GC-MS profile of ethyl acetate extract of *Streptomyces sp.* strain BN12

S.No	Compound Name	Retention Time	Chemical formula	Molecular weight	Biological activity	References
1	1-Butanone, 1-(2-hydroxyphenyl)	12.13	$C_{10}H_{12}O_2$	164.20	Food additive	(Beekwilder et al. 2007)
2	2,4- Monoethylidene-l-xylitol	13.47	$C_7H_{14}O_5$	177.66	No report	-
3	Isosativene	16.18	$C_{15}H_{24}$	204.35	Antibacterial, anticancer	(Chen et al. 2013; Park et al. 2016)
4	4,7-Methanoazulene, decahydro-1,4,9,9-trtramethyl	17.22	$C_{15}H_{26}$	206	No report	-
5	Flavone	17.93	$C_{15}H_{10}O_2$	222.24	Antimicrobial, anticancer, antioxidant	(Cushnie and Lamb 2005; Kopustinskiene et al. 2020)
6	Phytol	21.68	$C_{20}H_{40}O$	296.5	Food additive, antioxidant, anti-inflammatory, antimicrobial	(Islam et al. 2018)
7	Morin	25.35	$C_{15}H_{10}O_7$	302.23	Antioxidant, antidiabetic, anti-inflammatory, anti-tumor	(Caselli et al. 2016)
8	Octadecanoic acid, 3-oxo-, methyl ester	27.73	$C_{19}H_{36}O_3$	312.5	Antioxidant, anti-inflammatory, anticancer	(Kumar; Chenniappan et al. 2020)
9	2,6-Bis(1,1-dimethyl)-4-phenylmethylenecyclohexa-2,5-dien-1-one	19.52	$C_{21}H_{26}O$	294.4	No report	-
10	Oleic acid	18.92	$C_{18}H_{34}O_2$	282.46	Antioxidant, anti-inflammatory, anticancer	(Kumar)

Figures

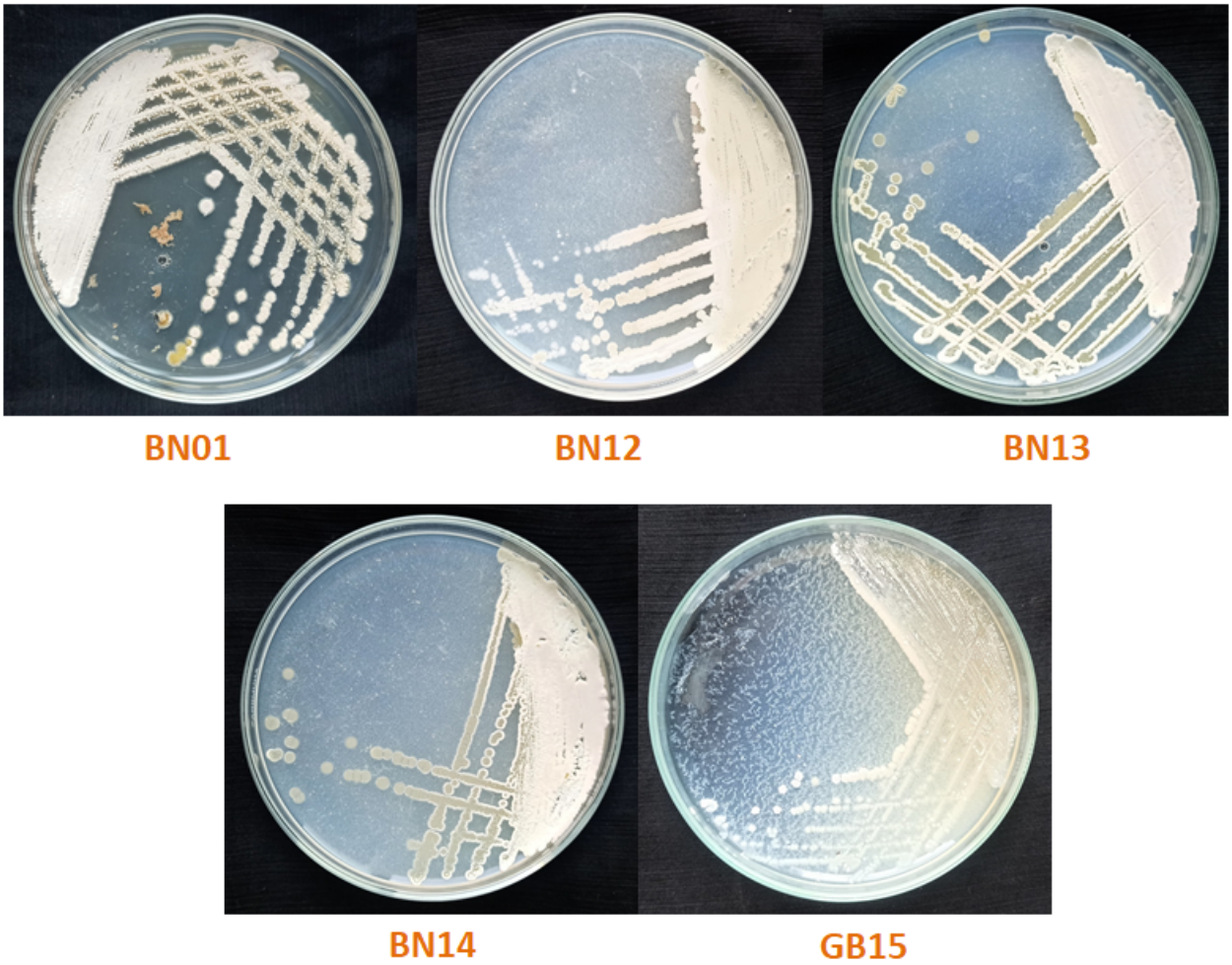

Figure 1

Colony morphology of marine actinomycetes isolates. The colonies appear in white and grey colour with powdery nature.

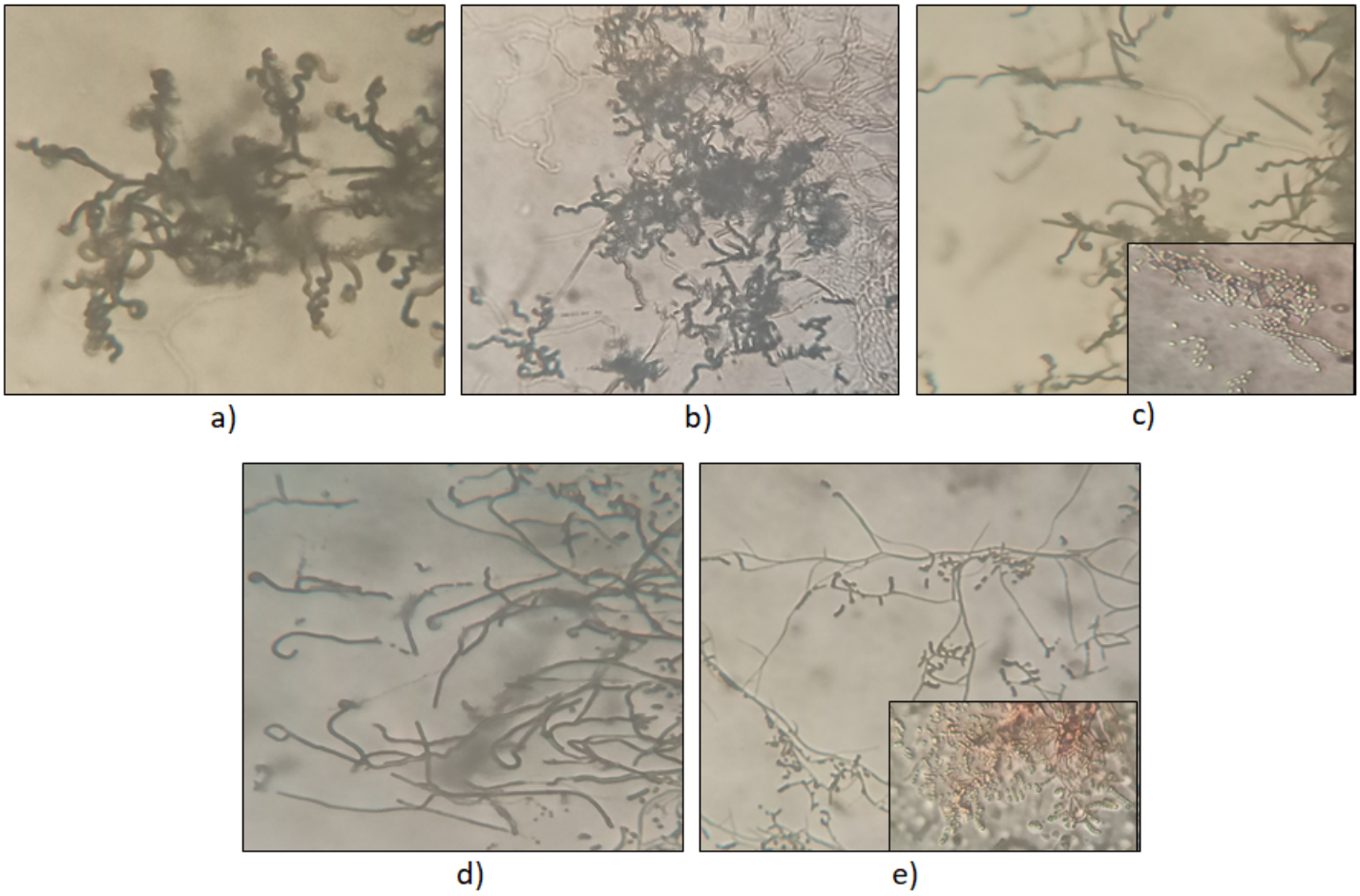

Figure 2

Light microscopy of actinomycetes isolates. a) BN01- Spira, **b)** BN12 – RA, **c)** BN13 – RA, **d)** BN14 - RA, **e)** GB15- RA. The isolates were observed at the magnification of 100x, 400x and 1000x which represents the structure of mycelium and spores.

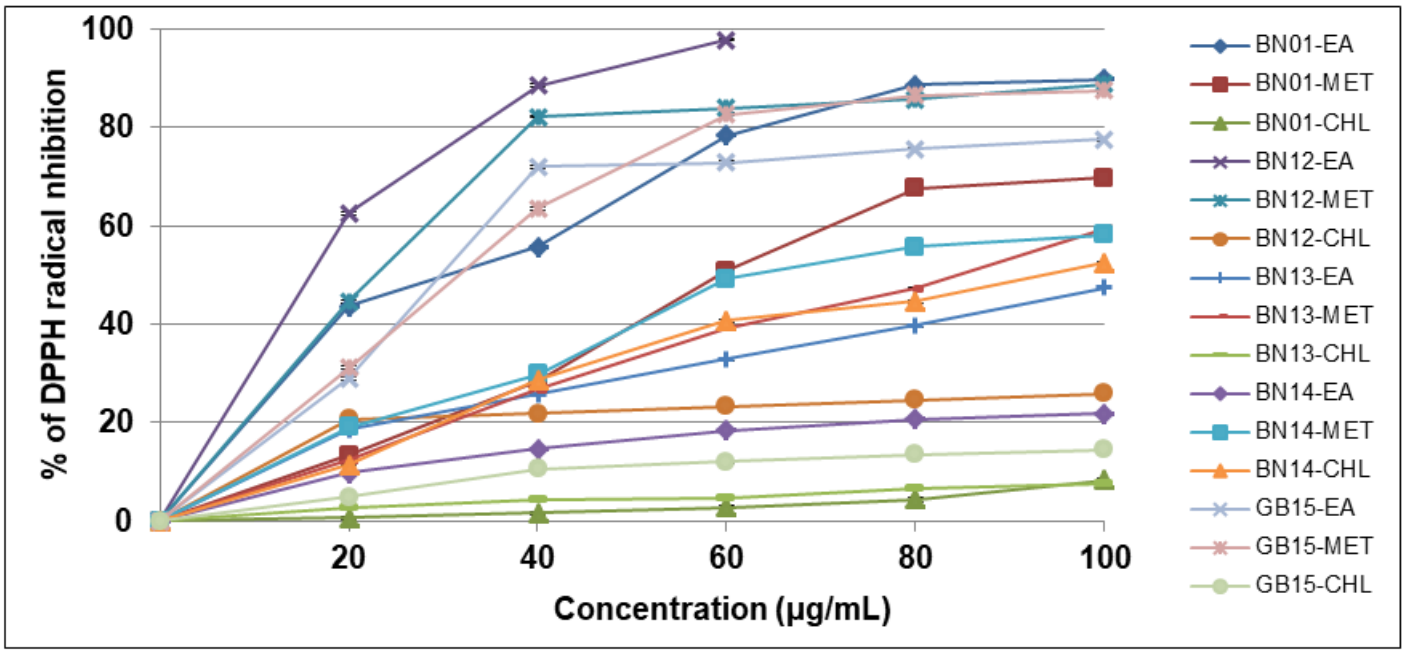

Figure 3

Percentage of radical scavenging activity of crude metabolite fractions. The graph plotted against concentration of crude extracts and % DPPH radical inhibition. Each sample represents in different colour. It shows that the isolate *Streptomyces sp.* strain BN12 - EA has increased radical scavenging activity of 97.75% at 60 µg/mL concentration (EA- Ethyl acetate, MET- methanol, CHL- chloroform).

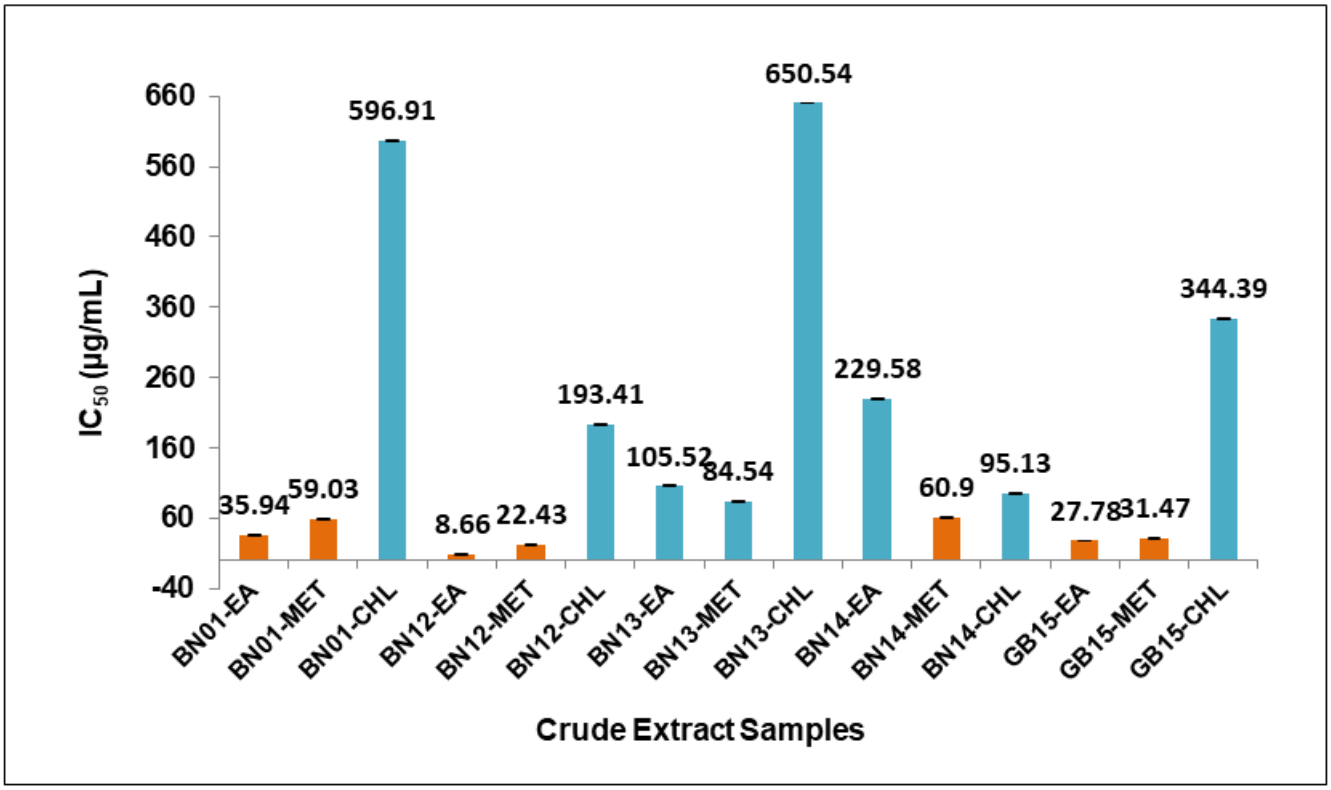

Figure 4

The IC_{50} value of the crude secondary metabolite fraction. The orange colour bar graph represents the extract samples having less IC_{50} values whereas blue colour graph represents the extract samples with high IC_{50} values. It shows that ethyl acetate extract of isolate *Streptomyces sp.* strain BN12 has the lower IC_{50} of 8.66 µg/mL. (EA- Ethyl acetate, MET- methanol, CHL- chloroform)

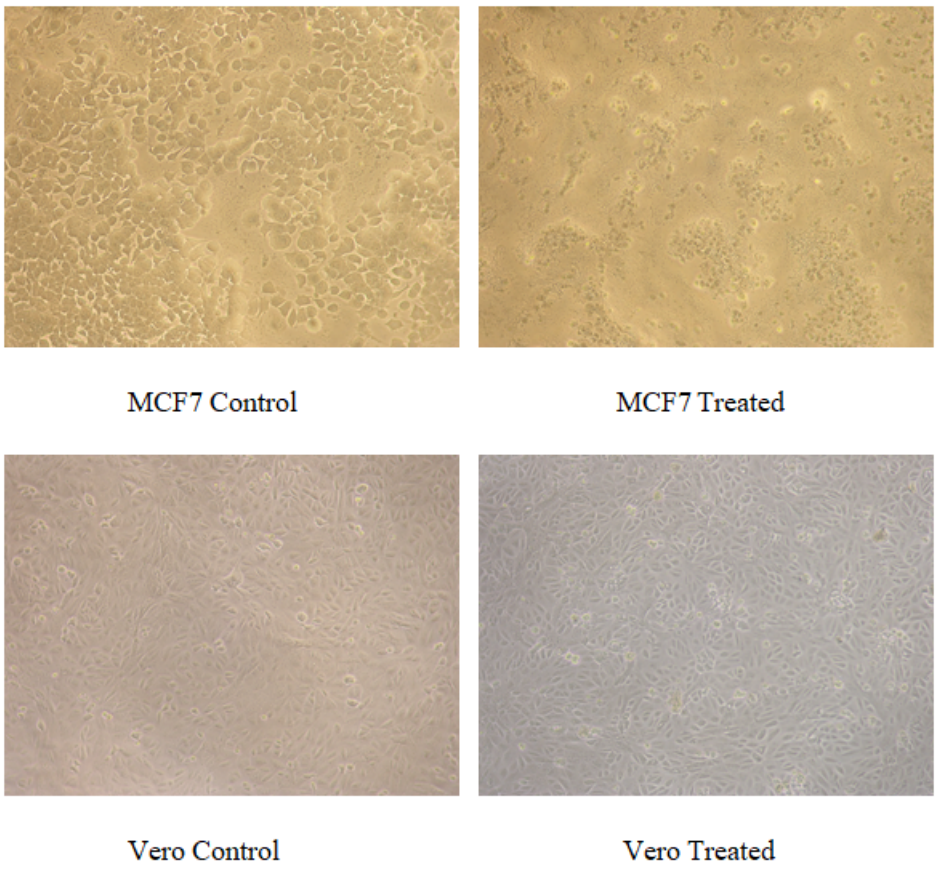

Figure 5

MTT- based cytotoxicity assay for crude extract of isolate *Streptomyces sp.* strain BN12. Morphological changes of MCF7 breast cancer cell line was observed after treatment with crude extracts of isolate *Streptomyces sp.* strain BN12 for 48h (100x magnification) and compared with the Vero cell line. Only less number of cells observed in MCF-7 treated whereas no changes observed in Vero treated cell line.

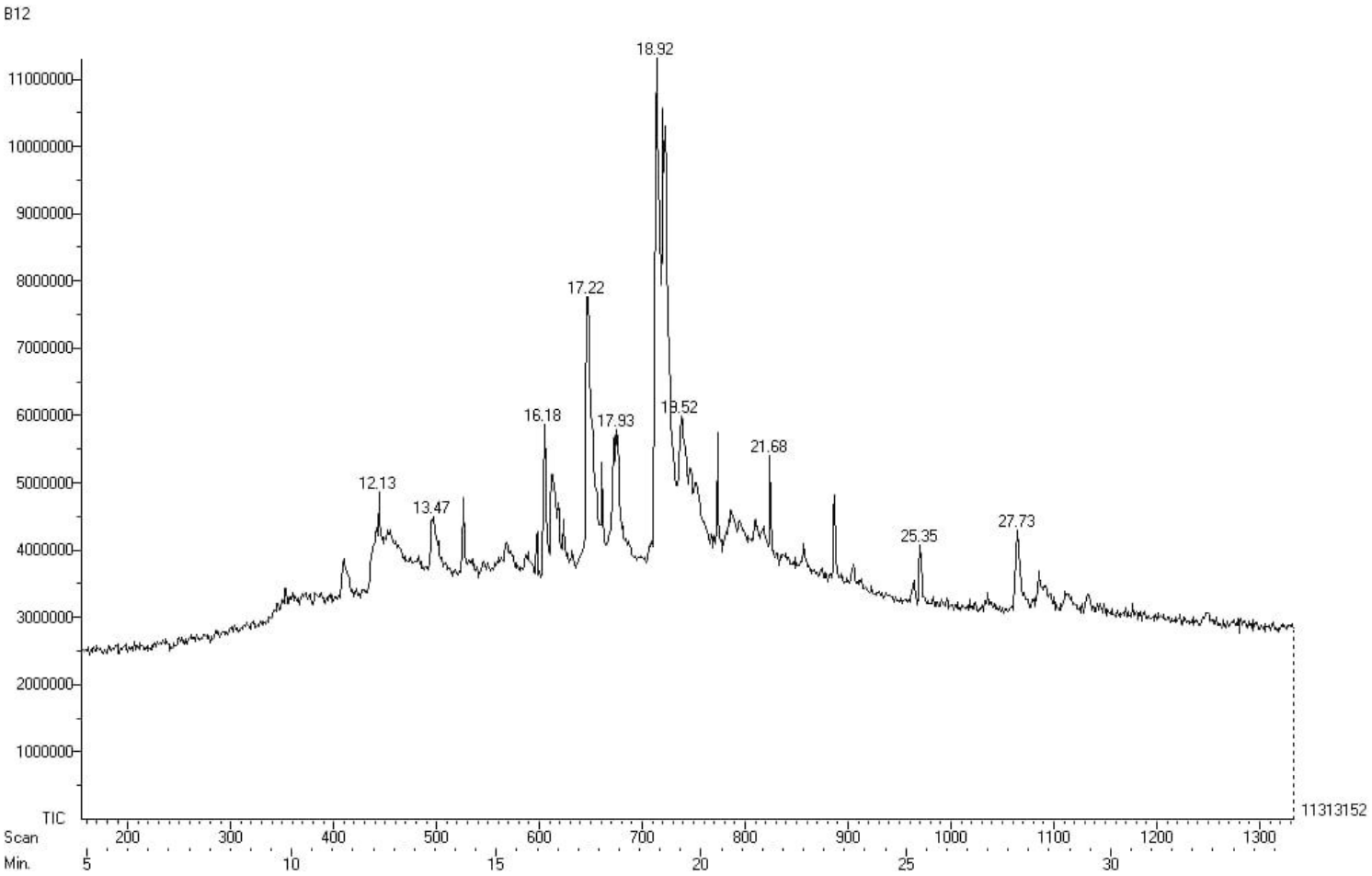

Figure 6

GC-MS analysis of ethyl acetate extract of *Streptomyces sp.* strain BN12. The compounds were identified by comparing with NIST library.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- floatimage1.jpeg