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Mechanism of action of antioxidant, antibacterial, antimicrobial and other agents

ANTICANCER AND ANTIMICROBIAL ACTIVITY OF MARINE ACTINOMYCETES

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ABSTRACT

The Actinomycetes are a phylum of mostly gram-positive bacteria. Actinomycetes can be found mostly in soil and decaying organic matter, as well as in living organisms such as humans and animals. They can also be seen in rhizosphere and non-rhizosphere soil. They can be terrestrial or aquatic. They are of great economic importance to humans because agriculture and forests depend on their contributions to soil systems. Many of the Actinomycetes have the ability to synthesise metabolites which hinder the growth of bacteria. Marine Actinomycetes are efficient producers of new secondary metabolites that show a range of biological activities including antibacterial, antifungal, anticancer, insecticidal and enzyme inhibition. Several aerobic Actinomycetes have been a major source of interest for the commercial drug industry and have proved to be extremely useful microorganisms for producing novel antimicrobial agents. They have also been well known as potential veterinary pathogens affecting many different animal species. Antimicrobial activity can be defined as a collective term for all active principles (agents) that inhibit the growth of bacteria, prevent the formation of microbial colonies, and may destroy microorganisms. A great number of antitumor compounds are natural products or their derivatives, mainly produced by microorganisms. In particular, Actinomycetes are the producers of a large number of natural products with different biological activities, including antitumor properties. Antitumor activity was studied by the MTT assay and DNA target activity was studied by the biochemical induction assay while antimicrobial activity was determined by observing bacterial and fungal growth inhibition. Marine organisms have attracted special attention in the last years for their ability to produce interesting pharmacological lead compounds.

Keywords: Actinomycetes, antimicrobial activity, anticancer activity.

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AIMS AND BACKGROUND

Actinomycetes are a diverse class of bacteria that produce novel bioactive compounds that have numerous therapeutic applications[1].. Actinomycetes have been isolated from various terrestrial ecosystems and are exploited for their bioactive potential and for antibiotic production[2]. Multidrug-resistant human pathogens and emerging infectious diseases are becoming a major threat to global health[3]. Actinomycetes are characterised under group of phylum Actinobacteria. They were distributed in different ecosystems especially soil ecosystem[4]. Recent studies using both traditional culture-based methods and advanced molecular techniques have shown the presence of native Actinomycetes in the oceans. These bacteria are widely distributed across various marine ecosystems, demonstrating their significance in the marine environment[5]. Actinomycetes are a type of Gram-positive bacteria that are commonly found in different environments. They are characterised by their filamentous morphology and high concentration of guanine and cytosine in their DNA (Ref. 6). The production of bioactive metabolites, including novel antibiotic compounds, is a key aspect of the organisms life cycle[7]. A significant portion of these natural products is produced during the transition from vegetative to aerial hyphal growth, particularly in Streptomyces species[8]. It has been estimated that one-third of naturally occurring antibiotics have been derived from Actinomycetes[9]. There is potential to discover new Actinomycetes with industrial and medical applications, including the production of enzymes and antimicrobials[10]. Marine microorganisms are gaining attention as a novel and prospective source of biologically active chemicals. They create a range of metabolites, some of which can be exploited in medication research. These microbes are potentially limitless sources of new chemicals with several medicinal uses. Actinomycetes are significant because of their wide diversity and shown ability to produce new compounds. Actinomycetes are recognised as the most commercially significant microorganisms, attributed to its ability to produce therapeutically and pharmaceutically vital compounds[11]. Actinomycetes are aerobic, Gram-positive bacteria that generate asexual spores. It has been assumed to be a fungus or a bacterium, but it is now identified as a prokaryotic species. They are branching unicellular microorganisms. The actinobacteria phylum Actinomycetes is characterised by a greater G + C content (70–80%). Among the Actinomycetes, Streptomyces species manufacture over 70% of naturally available antibiotics and generate metabolites with antiparasite, antibacterial, anticancer, antiviral, and other pharmaceutically useful action. Streptomyces has recently been identified as the "modern Actinobacteria" (MOD-ACTINO), which consists of a group of actinobacteria able to produce components that may be studied for modern uses such as therapeutic drugs and cosmeceuticals. The majority of Actinomycetes live both in aquatic and terrestrial environments. Recently, researchers conducted extensive screenings of Actinomycetes on marine plants, medicinal plants, sediments, and soil habitats in order to identify their bioactive compounds. The most commercially and biotechnologically important prokaryotes are Actinomycetes[12]. They create almost half of the bioactive secondary metabolites known. Microorganisms have been docu-

mented to create around 23 000 bioactive secondary metabolites, with Actinomycetes producing over 10 000 of these chemicals, accounting for 45% of all bioactive microbial metabolites known. Among Actinomycetes, Streptomyces species produce over 7600 compounds. The Marinlit database comprises 289 secondary metabolites from the marine-derived class Streptomyces, including peptides, macrolides, lactones, indoles, terpenes, and quinones. These chemicals have a wide range of mechanically beneficial qualities, such as cytotoxicity, antibacterial, antifungal, antimalarial, anticancer, immunosuppressive, anti-inflammatory, anthelmintic, herbicide, protein, and others. They play a crucial part in the carbon cycle and the recycling of organic matter through the degradation of organic substances, such as chitin and cellulose. Phosphate solubilisation, chitinolysis, lipolysis, amylolysis, and proteolytic activity have all been documented in marine actinobacteria. Many different types of cytotoxic chemicals that function as anticancer substances are produced by marine actinobateria. Especially in the context of Actinomycetes in a variety of fields, the current work aimed to isolate Actinomycetes from a unique source[11]. In addition, the most potent isolate was identified and its antibacterial and anticancer properties were evaluated.

EXPERIMENTAL

Source of Actinomycetes. The samples were collected from the soil sediments from the coastal region of Tamil Nadu, India. The isolated marine Actinomycetes were obtained from the Armats Biotek Training and Research Institute, Guindy, Chennai. There were five isolated marine Actinomycetes, namely: BN01 – *Streptomyces rochei* (NCBI accession No: ON042751); BN12 – *Streptomyces species* (NCBI accession No: yet to be received); BN13 – *Streptomyces species* (NCBI accession No: ON042452); BN14 – *Streptomyces species* (NCBI accession No: ON042463); BN 15 – *Saccharopolyspora species* (NCBI accession no: ON042476) (Fig. 1). All five isolates were used for determination of their antimicrobial and anticancer activities. The isolated Actinomycetes were sub-cultured in the starch casein agar medium (Table 1).

Table 1. Preparation of starch casein agar medium

Components	Volume for 1 l
Starch	10 g
Vitamin free casein	0.3 g
KNO_3	2 g
NaCl	2 g
$MgSO_4$	2 g
K_2HPO_4	2 g
$CaCO_3$	0.02 g
$FeSO_4$	0.01g
Distilled water	1 l

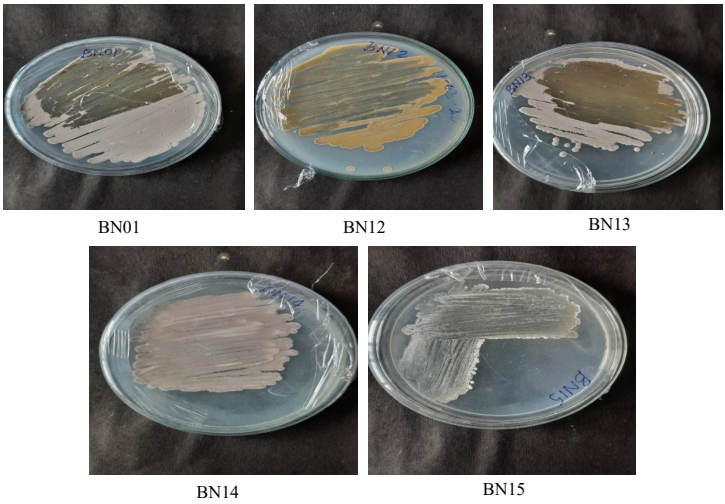

Fig 1. Actinomycetes growth in starch casein agar medium

The starch casein agar medium was autoclaved for 30 min. Starch casein agar medium was used to identify saccharolytic marine microorganisms and used for the isolation of Actinomycetes.

Result interpretation of starch casein agar medium: Actinomycetes present in the medium show off-white to yellow-coloured homogenous free-flowing powder.

Preparation of ISP-2 medium. ISP-2 medium is also known as yeast extract-malt extract medium. ISP-2 medium was prepared according to Table 2.

Table 2. Preparation of ISP-2 medium

Components	Volume for 1 l
Yeast extract	4 g
Malt extract	10 g
Dextrose	4 g
Agar	20 g
Distilled water	1 l
pH	7.3

ISP-2 medium was autoclaved for 30 min and cooled for 10 min. In ISP-2 medium one loop full of cultured bacteria was inoculated into 5 conical flasks and was kept in the shaker for 3 days to grow.

Preparation of production media. The grown bacteria in the ISP-2 medium were inoculated into the production media to produce a large number of bacteria (Table 3).

Table 3. Preparation of production media

Components	Volume for 1 l
Glycerol	7 ml
Glucose	3 g
Beef extract	3 g
Peptone	0.8 g
Sodium nitrate	0.2 g
$MgSO_4$	0.01 g
$CaCO_3$	0.3 g
K_2HPO_4	0.25 g
KH_2PO_4	0.25 g
Distilled water	1000 ml
pH	7
NaCl (only for marine bacteria)	0.75 g

The prepared production media was kept in an autoclave for 30 min and cooled at room temperature. In the production media, ISP-2 media was poured and kept in shaker for 7 days to grow. After 7 days, the production media was centrifuged and the supernatant was collected and stored.

RESULTS AND DISCUSSION

EXTRACTION OF SECONDARY METABOLITES

The culture filtrate was extracted three times with ethyl acetate as the solvent. The solvent was added to the filtrate in a 1:1 ratio and forcefully agitated for 20 min. Using a separating funnel, the antibiotic-containing ethyl acetate phase was separated from the aqueous phase. The ethyl acetate layer was concentrated by evaporation to dryness at 40°C, and the residue was purified with methanol to yield (0.8 g) of dark crude extract. This compound was tested for antibacterial activity against pathogenic bacteria using the agar-well diffusion technique. Similarly, all the samples were extracted through liquid-liquid extraction[13].

ANTIBACTERIAL ACTIVITY

The discovery of actinomycete isolates with selective antibacterial and antifungal activities indicates the potential production of a variety of antimicrobial secondary metabolites and the secretion of compounds with a wide range of targets against microorganisms[14]. To evaluate their antibacterial properties, the Actinomycetes would be cultivated in a specialised growth medium and tested for their ability to inhibit the growth of Gram-positive and Gram-negative bacteria[15]. The crude methanol and ethyl acetate extracts named BN01, BN12, BN13, BN14, GB15 were evaluated for antibacterial activity using the agar-well diffusion technique, by swabbing 25 µl of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneu-*

moniae on Muller-Hinton agar. *Staphylococcus aureus* and *Enterococcus faecalis* are Gram-positive bacteria whereas *Escherichia coli* and *Klebsiella pneumoniae* are Gram-negative bacteria. The wells were dug with the help of the sterile 6 mm cork borer and 100 µl of the crude metabolite extract are loaded into the well. The plates were kept at 4°C for 12–16 h before being incubated at 37°C overnight. The inhibition zone of test microorganisms was determined around the wells[13]. For antibacterial activity (Table 5) tetracycline was used as the control in Mueller-Hinton (MH) agar medium (Table 4).

Table 4. Preparation of Mueller-Hinton agar medium

Components	Volume
Beef extract	3.0 g
Peptone	17.5 g
Starch	1.5 g
Distilled water	1000 ml
Agar	20 g
pH	7.3

Table 5. Antibacterial activity of the isolated strains of Actinomycetes

S.No	Actinomycetes strains	*Staphylococcus aureus*	*Enterococcus faecalis*	*Escherichia coli*	*Klebsiella. pneumoniae*
1	BN01(EA)	–	21 mm	–	16 mm
2	BN12(EA)	10 mm	–	10 mm	12 mm
3	BN13(EA)	–	23 mm	–	–
4	BN14(EA)	–	15 mm	–	–
5	BN15(EA)	–	–	–	–
6	BN01(M)	–	17 mm	–	–
7	BN12(M)	–	–	–	–
8	BN13(M)	–	–	–	–
9	BN14(M)	–	–	–	–
10	BN15(M)	–	–	–	–
	Control	26 mm	24 mm	29 mm	28 mm

ANTIFUNGAL ACTIVITY

Marine Actinomycetes are being studied as a source of new antifungal compounds due to their potential to produce these compounds[16]. Many species of Actinomycetes, especially those in the genus streptomyces, have been recognised as effective antifungal agents for biocontrol purposes[17]. The crude methanol and ethyl acetate extracts named BN01, BN12, BN13, BN14, GB15 were evaluated for antifungal activity using the agar-well diffusion technique, by swabbing *Candida albicans*, *Candida tropicalis* and *Candida krusei* on potato dextrose agar medium (Table 6). The wells were dug with the help of the sterile 6 mm cork borer and 100 µl of the crude metabolite extract were

loaded into the well. The plates were kept at 4°C for 12–16 h before being incubated at 37°C overnight. The inhibition zone of test microorganisms was determined around the wells. For antifungal activity (Table 7) fluconazole was used as a control in potato dextrose agar medium.

Table 6. Preparation of potato dextrose medium

Components	Volume
Potato	200 g
Dextrose	20 g
Distilled water	1000 ml
Agar	20 g
pH	6.5

Table 7. Antifungal activity of the isolated strains of Actinomycetes

S. No.	Actinomycetes strains	*Candida albicans*	*Candida tropicalis*	*Candida krusei*
1	BN01(EA)	13 mm	–	14 mm
2	BN12(EA)	10 mm	–	11 mm
3	BN13(EA)	10 mm	–	–
4	BN14(EA)	–	–	–
5	BN15(EA)	–	–	–
6	BN01(M)	–	–	12 mm
7	BN12(M)	–	–	11 mm
8	BN13(M)	–	–	–
9	BN14(M)	11 mm	–	–
10	BN15(M)	10 mm	–	12 mm
	Control	27 mm	28 mm	28 mm

ANTICANCER ACTIVITY

Cardiovascular disease is the leading cause of both death and illness globally[18]. Cancer is a significant contributor to global mortality, ranking as second leading cause of death worldwide. In 2018, it was estimated that cancer was responsible for roughly 9.6 million deaths. This equates to approximately 1 in 6 deaths caused by cancer. It is a widespread issue with far-reaching impacts on individuals, families, and communities[19]. In this research BN01 sample was evaluated for anticancer activity against breast cancer cell line (MCF-7) cell lines and the cytotoxicity to the normal cell line was also studied. The IC_{50} values of the sample against cancer cells were found to be better.

MTT-BASED CYTOTOXICITY ASSAY

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[20]. The MTT assay is a method used to assess the viability of cells by determining their ability to convert a soluble tetrazolium salet called

MTT, into an insoluble formazan precipitate[21]. The MTT assay is widely used in the evaluation of cell toxicity, however it is often used and interpreted errorneously[22]. In the further study anti-platelet activity will be performed for various biological applications from Actinomycetes[23]. The cytotoxic effect of compounds against human tumour cell lines was determined by a rapid colorimetric assay, using 3-(4,5 dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and compared with untreated controls. For the screening experiment, the cells were seeded in 96-well plates in 100 µl of medium containing 5% FBS, at plating density of 10 000 cells/well and incubated at 37°C. Triplicates were maintained and the medium without the sample served as a control. The cells were maintained at 5% CO_2, 95% O_2, and 100% relative humidity for 48 h. After 48 h, 50 µl of MTT (5 mg/ml) in triple distilled water were added to each well and incubated at 37°C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilised in 100 µl of DMSO and then measured the absorbance at 570 nm using microplate reader. The percentage of cell inhibition was determined using the following formula:

$$\text{cell inhibition (\%)} = 100 - \text{Absorbance (Sample)/Absorbance (control)} \times 100.$$

CONCLUSIONS

In conclusion, Actinomycetes are still an important source of bioactive compounds that are used for treating infectious diseases, cancer, and many other diseases. Considering the enormous biological variety of the sea as a whole, it is becoming increasingly clear that the seas include a substantial number of unique chemical substances. Because marine microorganisms, particularly Actinomycetes, have developed with the highest genetic and metabolic diversity, efforts should be aimed at discovering new secondary metabolites from marine Actinomycetes. Actinomycetes are found not just in the oceans, but also in a variety of marine environments. The use of marine Actinomycetes as a source of new secondary metabolites is in its early stages. Though many isolated bioactive strains mentioned above have been reported before were derived from marine environment. Thus, the present study demonstrated that Actinomycetes from marine environment are a rich source for disclosure of antibiotics and anticancer properties.

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