

Antimicrobial, Antioxidant and Cytotoxic Activity of Marine *Streptomyces* MS-60 Isolated from Bay of Bengal

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Abstract: The main aim of the study was to evaluate the antimicrobial, antioxidant and cytotoxic potential of crude extract of Marine *Streptomyces* species isolated from soil of Chennai coast, Tamilnadu. Primary and Secondary screening for antimicrobial activity was determined by Cross streak method and Agar well diffusion method respectively. Gram positive bacteria were found most sensitive to the actinomycetes isolate MS-60 in both primary and secondary screening. The actinomycete isolate was identified as a species of the genus *Streptomyces* on the basis of microscopic and biochemical characteristics. The compounds present within the crude extract of Marine *Streptomyces* MS-60 showed maximum antimicrobial activity against *Streptococcus mutans* (34mm), and minimum activity against *Klebsiella pneumonia* (13 mm). Antioxidant activity of the crude extract of MS-60 was tested by 1, 1-Diphenyl-picrylhydrazyl (DPPH) free radical scavenging assay and it exhibit potent scavenging activity at (Ic₅₀12 µg/ml). The anticancer activity of the secondary metabolite of actinomycetes was evaluated on HT-29 (Human colon cancer cell) line by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The *in vitro* cytotoxicity assay on Human colon cancer cell line [HT-29] revealed that the secondary metabolite had the strongest cytotoxicity with Ic₅₀26.5µg/ml.

Keywords: Marine *Streptomyces* sp., Antimicrobial activity, Antioxidant activity, MTT, Cytotoxicity.

1. Introduction

The development of drug resistance by the pathogens is due to the concurrent usage of existing antibiotics. The search for new, safer, broad-spectrum antibiotic with greater potency has been progressing slowly [1]. New antibiotics should be developed to combat these drug resistant pathogens. There is no satisfactory drug as of now to control the occurrence of cancer and it is known to be the leading cause of death worldwide [2]. The limited availability to control cancer drug resistance along with the emergence of new pathogens has led the expansion of the search for novel drug candidates. In the past thirty years, several researchers have demonstrated that marine organisms, especially marine invertebrates are capable of producing cytotoxic compounds [3-5]. However, marine microorganisms are understudied and only a few cytotoxic compounds have been reported so far. The examples are palau'amide [6], salinosporamide A [7], micromide [8], resistoflavine [9], and trichoderamide B [10]. Interestingly, cytotoxic compounds from marine microorganisms have been produced mainly by marine fungi and actinomycetes.

Actinomycetes are the most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites notably antibiotics [11] anti tumor agents, immunosuppressive agents [12] and enzymes [13-14]. A number of structurally unique natural products with antitumor [15-16] anti-infective [17] and antimalarial bioactivities [18] have been discovered from marine-derived actinomycetes. The species of *Streptomyces* are aerobic spore formers and possess DNA rich in GC content (69-73 %) *Streptomyces* species are filamentous and they form extensive branching substrate and aerial mycelia. They are considered as prolific producers of bioactive compounds as they produced around 75% of biologically active compounds. [19-20]. Indian coastal region is a best location

for obtaining many numerous species producing bioactive compounds which exhibit lesser side effects when compared to medical drugs. The discovery of new active metabolites must be followed by adequate biological testing [21]. Hence, the aim of this study was to investigate the bioactive potential of crude extracts of Marine *Streptomyces* MS-60 from marine environment of Bay of Bengal, Chennai coast of India for its antibacterial, antioxidant and cytotoxic activity.

2. Materials and Method

2.1 Collection of Soil Sample

Soil samples were collected from Ennore (Lat.13°.14' N, Long. 80°.22' E), Muttukadu estuary (Lat. 13°.59' N, Long. 80°.15' E), Marina Beach, Chennai (Lat. 13°.54' N; Long. 80°.28' E) in the coastal region of the Bay of Bengal, India. The soil samples were taken from the 20 cm depth after removing approximately 3 cm of upper soil surface. The samples were placed in the polythene bags, closed tightly and stored in a refrigerator. The processed samples were given proper identification code. [22]

2.2 Isolation and Screening of Actinomycetes:

Collected soil samples were treated with 2% calcium carbonate and air dried for 3 to 4 days under *in vitro* lab condition. The soil samples were serially diluted from 10⁻¹ to 10⁻⁷ and 100µl of diluted samples were inoculated in Starch Caesin agar prepared with 50% marine water by spread plate technique. The media was supplemented with flucanazole to avoid fungal contamination. The plates were incubated at 30°C for 7-10 days. After incubation period actinobacteria colonies were separated and purified from mother plate [23]. Preliminary screening was done using cross-streak method [24] by streaking Actinomycetes isolates in the centre of

starch casein agar plates. After incubation at 28°C for 6 days, 24 hrs cultures of MTCC pathogens *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas sp.*, *Klebsiella pneumonia*, *Vibrio cholerae*, *Streptococcus mutans*, *Proteus vulgaris* and *Mrsa* were streaked perpendicular to the central strip of actinomycetes culture. All plates were again incubated at 30°C for 24 hrs and inhibition was measured in millimeters (mm).

2.3 Identification of potential actinomycetes

The spore chain morphology such as sporangia spore motility and spore surface ornamentation of the isolate was evaluated by phase contrast microscope magnifications 100x. This is done by using cover slip method [25]. The generic level identification of potential actinomycetes was carried out. The morphological, cultural, physiological, biochemical, colour and carbon utilization were carried out using standard procedure and were identified based on the keys of Bergey's manual of determinative bacteriology [26].

2.4 Production of secondary metabolites:

The potential actinomycete MS-60 was inoculated in 500ml Erlenmeyer flask 1000ml into the production medium containing 50% sea water and incubates at 28°C in shaker (200-250 rpm) for 5 days. The production media (ISP-2) consist of Malt extract 10gm, Yeast extract 4 gm, Glucose 10gm, Peptone 5gm in 1000 ml of distilled water with 7.2 pH. These inoculums were transferred to 500ml medium and incubated for 10-15days at 28°C in shaker (200-250 rpm).

2.5 Extraction of bioactive metabolites:

After 15 days, the culture was taken and centrifuged at 3000-5000 rpm for 10 minutes. After centrifugation, the cell free supernatant was collected and filtered with Whatmann no.1 filter paper. To the supernatant equal volume of ethyl acetate was added in the ratio of 1:1 in a separating funnel, shaken well for 1 hr and allowed to settle overnight in a stand. The upper aqueous layer containing the bioactive compound was collected in watch glass and kept in hot air oven at 40°C for 4 hours.

2.6 Antibacterial activity of crude metabolites against MTCC pathogen:

The dried compound was scrapped, mixed with distilled water and antibacterial activity was seen by well diffusion method against pathogenic organisms obtained from MTCC, Chandigarh, India. The organisms used were gram positive bacteria *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 1538), *Streptococcus mutans*(MTCC 497), *Shigella flexneri* (MTCC 1457), *Bacillus cereus* (MTCC 441) and gram negative bacteria *Escherichia coli* (MTCC 443), *Pseudomonas sp.*, (MTCC 129), *Proteus vulgaris* (MTCC 426), *Klebsiella pneumonia* (MTCC 109). The extracted bioactive compound mixture was added to each well at different concentration (25 µl, 50 µl, 75 µl, 100 µl) and Tetracycline was taken as a control. The plates were incubated at 37°C for 24 hours and the diameters of inhibition zones were measured.

2.7 Antioxidant activity by DPPH (1, 1-diphenylpicrylhydrazyl) radical scavenging assay :

5ml of 0.2mM DPPH solution was prepared using methanol and added 1ml of sample solutions. The mixture was vortex-mixed and incubate in dark at 37°C for 30 mins. The optical density (OD) was measured at 517nm. Methanol was used as baseline control [27]. The experiments performed in triplicates. The capability to scavenge the DPPH radical was calculated using the following equation

$$\text{DPPH radical scavenging effect (\%)} = \left[\frac{\text{Control} - \text{Test sample}}{\text{Control}} \times 100 \right]$$

2.8 Cell lines and culture conditions:

The human colon cancer cell line (HT-29) was obtained from the NCCS -Pune. The cells were cultured in McCoy's 5A containing 10% heat-inactivated FBS and 100 units/mL penicillin, in a humidified atmosphere of 5% CO₂-air at 37°C. Cell densities did not exceed 1 × 10⁶ cells/mL.

2.9 Cytotoxicity determination by MTT assay:

The effect of crude compound on cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) MTT in cultured HT-29 cells. HT-29 cells were seeded at a concentration of 5 × 10⁵ cells/well in 96-well plates and incubated at 37 °C in 5% CO₂ for 24 h. Once the cells reached 70% confluency, media with different concentrations of compound (1, 10, 25, 50, 75, 100, 500, 1000 µM) were added and incubated at 37 °C in 5% CO₂ for 24 and 48 h. The samples also included a 'blank' (medium alone) and 'control' (DMSO alone). After 24 and 48 h, the absorbance recorded at 570 nm using microtiter plate reader. The percentage of growth inhibition (IC₅₀) was determined as described by Mosmann [28] and Tyson and Green [29], respectively.

The percentage viability was calculated as follows:

$$\text{Cell Viability} = \frac{\text{Optical density of samples}}{\text{Optical density of control}} \times 100$$

3. Results

3.1 Isolation and Identification of Actinomycete Isolate:

The Indian subcontinent has an immense biological diversity and it is increasingly recognized that a large number of chemical entities exists as metabolites in the micro flora. Actinomycetes have evolved as a group with greatest genomic and metabolic diversity [30]. In this course of study 63 isolates were isolated from Ennore, Muttukadu, Marina soil sample. Out of 63 actinomycetes isolates were subjected to primary screening process, 16 isolates showed inhibition against both Gram positive and Gram negative bacterial pathogens. The primary screening of potential actinomycete isolate MS-60 was shown in [Fig.1] and their inhibition values are shown in Table.1 The selected potential actinomycete MS-60 was viewed under microscope [Fig-2] and was identified as *Streptomyces species* based on the keys of Bergey's manual of determinative bacteriology.



Figure 1: Primary screening of MS-60 against MTCC bacterial pathogens



Figure 2: Microscopic view Of Marine Streptomyces MS-60

Table 1: Primary screening of *Streptomyces* sp. MS-60 against MTCC bacterial pathogens

Pathogens	Inhibition Zone (Mm)	Pathogens	Inhibition Zone (Mm)
<i>Staphylococcus aureus</i>	14	<i>Klebsiella pneumonia</i>	05
<i>Micrococcus luteus</i>	11	<i>Pseudomonassp</i>	10
<i>Bacillus cereus</i>	13	<i>Vibrio cholarae</i>	10
<i>Streptococcus mutans</i>	11	Mrsa1	02
<i>Escherichia coli</i>	10	Mrsa2	01
<i>Proteus vulgaris</i>	09	Mrsa3	-

The cultural, morphological, physiological and biochemical characteristics of the isolate has been shown in Table 2.

Table 2: Morphological, biochemical and physiological characteristics of the Marine Streptomyces MS-60

Characteristics	Results
Spore chain morphology	Spiral open
Spore surface	Smooth
Color of aerial mycelium	Pure White
Color of substrate mycelium	Dark Brown
Gram staining	Positive
Biochemical tests	

Nitrate	Positive
Starch	Positive
Gelatin liquefactions	Positive
Milk coagulation	Positive
Soluble pigment	Positive
Carbon utilization	
D-glucose	Positive
L-arabinose	Positive
Fructose	Positive
Maltose	Positive
Mannitol	Positive
Sucrose	Negative
Physiology	
Growth at temperature	30-40° c
Growth at pH	7-9

3.2 Extraction of Bioactive Metabolites:

The marine streptomyces MS-60 was inoculated in ISP-2 medium showed good culture growth and pigment production was observed on 5 th day after incubation shown in [Fig.3]. The supernatant containing the metabolites was extracted with equal volumes of ethylacetate in a separating funnel shown in [Fig.4] and the compound residue were collected and evaporated.



Figure 3: Culture Growth Of Ms-60



Figure 4: Extraction Of Bioactive Metabolite

3.3 Bioactivity of the Crude Metabolites

3.3.1 Determination of Antimicrobial Activity

The antimicrobial activity of crude metabolites of marine *Streptomyces* sp. MS-60 against MTCC bacterial pathogens was determined using well diffusion method. The crude metabolites have shown excellent antimicrobial activity against *Streptococcus mutans* (34±0.3mm), *Micrococcus*

luteus and *Staphylococcus aureus* (22±0.1mm), *Proteus vulgaris* (21±0.3mm), *Escherichia coli* and *Shigella Flexneri* (20±0.1mm), *Bacillus Subtilis* (18±0.2mm), *Pseudomonas sp.*, (17±0.2mm) and *Klebsiella pneumoniae* (13±0.1mm). [Fig.5]. The crude metabolites of marine *Streptomyces* sp. MS-60 showed more inhibitory activity against Gram positive organisms than Gram negative organisms and they are shown in [Table 3].

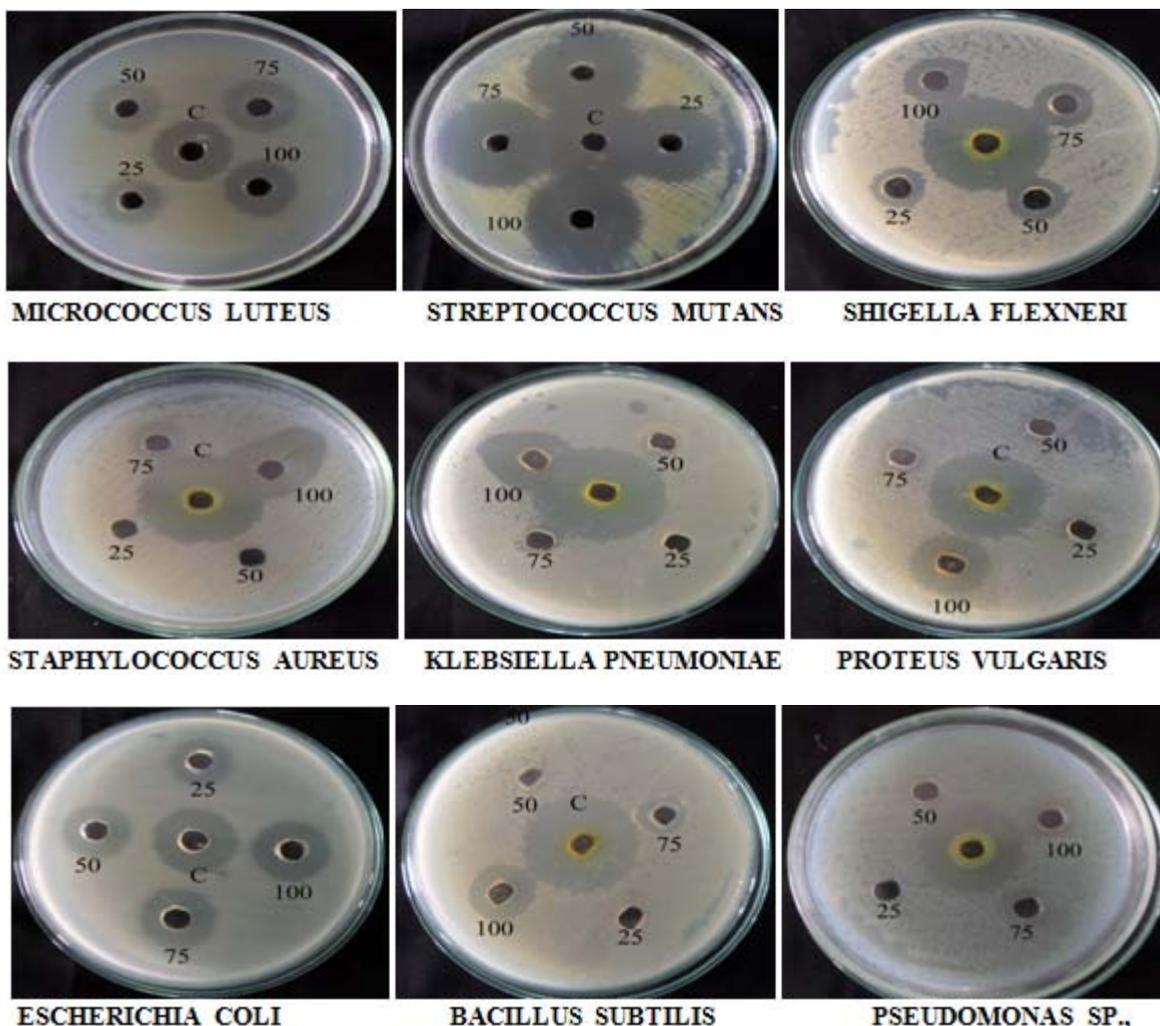


Figure 5: Antibacterial activity of crude bioactive compound of Marine *Streptomyces* sp MS-60

Table 3: Antibacterial activity of crude bioactive compound of Marine *Streptomyces* MS-60

S.NO	MTCC Pathogens	Diameter Zone of Inhibition in (Mm)				Standard Antibiotic 100 µl
		25 µl	50 µl	75 µl	100 µl	
1	<i>Streptococcus mutans</i>	26	29	32	34	36
2	<i>Pseudomonas</i>	06	11	13	17	30
3	<i>Micrococcus luteus</i>	13	17	20	22	25
4	<i>Proteus vulgaris</i>	-	10	12	21	30
5	<i>E.coli</i>	14	17	19	20	23
6	<i>Staphylococcus aureus</i>	7	12	18	22	35
7	<i>Shigella Flexneri</i>	10	12	16	20	33
8	<i>Klebsiella pneumoniae</i>	-	06	08	13	31
9	<i>Bacillus Subtilis</i>	-	07	13	18	32

3.3.2 Antioxidant activity of MS-60

Free radicals are chemical species containing one or more unpaired electrons that make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [31]. Antioxidant activity of crude extract of Marine *Streptomyces* sp MS-60 exhibit potent scavenging activity (Ic₅₀ 12 µg/ml) [Fig. 6]

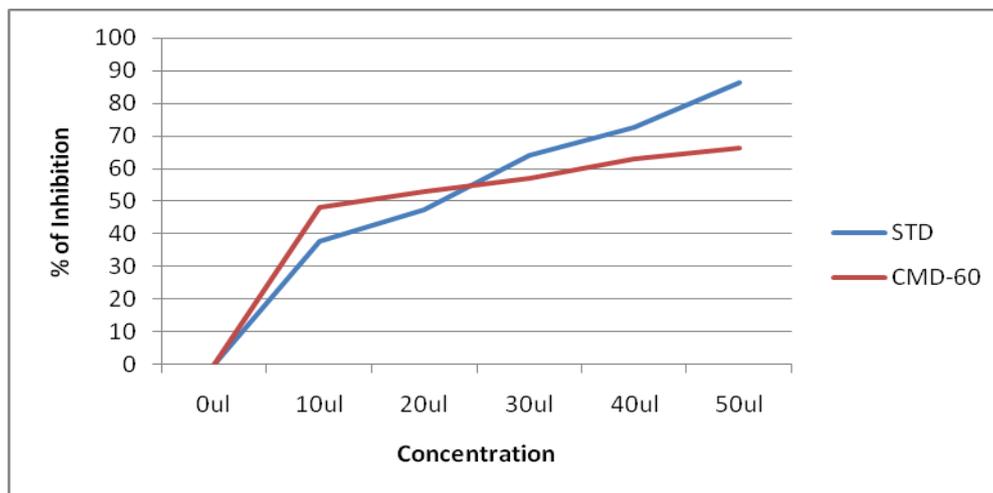


Figure 6: DPPH Assay of crude extract of Marine *Streptomyces* sp MS-60

3.3.3 Cytotoxicity effects of Marine *Streptomyces* extracts

The anticancer activity of the secondary metabolite of marine streptomyces MS-60 was evaluated on HT-29 (Human Colon Cancer cell line) by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity effects of the extracts were increased with the

increase of the extracts concentration. Also, the results demonstrated that the average cytotoxicity effect of *Streptomyces* was 63.9% cell inhibition for 75 μ l of the crude extract of *Streptomyces* MS-60 shown in the graph [Fig.7].

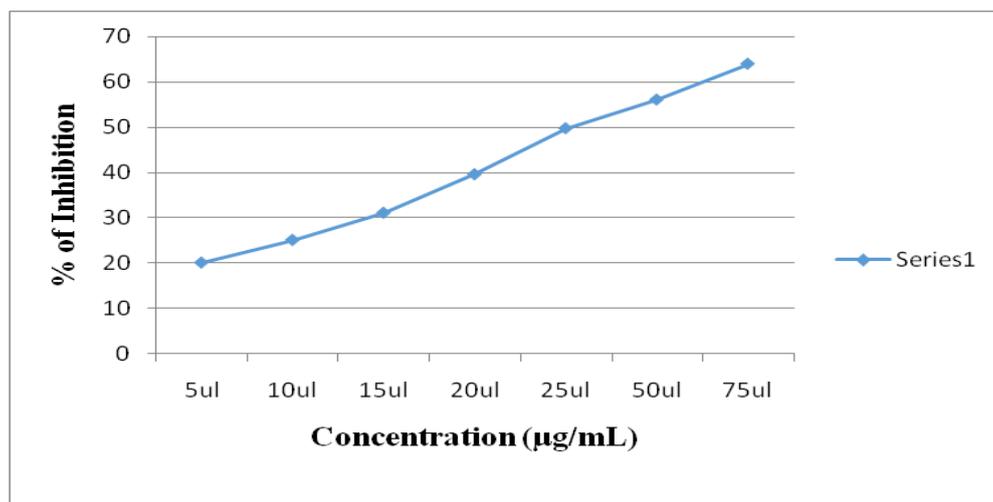


Figure 7: MTT Cytotoxicity activity of the crude extract on HT-29 cell lines

The in vitro cytotoxicity assay on Human Colon Cancer cell line revealed that the secondary metabolite had the cytotoxicity with IC50 of 26.5 μ g/ml. [Fig.8]

These results suggest that crude compound from *Streptomyces* MS-60 was toxic to HT-29 cells.

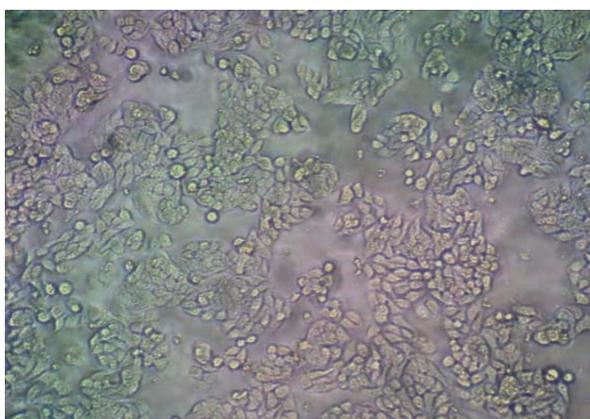
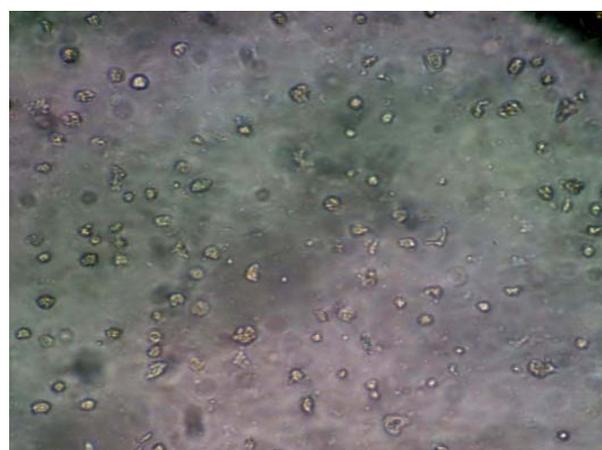


Figure 8: a) HT-29 cell line control



b) HT-29 cell line treated with crude extract

4. Discussion

Microorganisms are attractive sources of biologically active compounds having pharmaceutical and agricultural significance. These actinomycetes are biotechnologically and industrially valuable prokaryotes as they produce a large number of bioactive compounds with pharmaceutical and agricultural importance [32]. In the present study, we have recovered 63 actinomycete isolates from a marine soil sample from Ennore, Muttukadu estuary, Marina Beach, in the coastal region of the Bay of Bengal, India. Out of 63 isolates, MS-60 isolates displayed marked inhibitory activity against MTCC bacterial pathogens in primary screening by cross streak technique. The isolate was characterized as a species of the genus *Streptomyces* on the basis of microscopic and biochemical characteristics. This study evaluated the antibacterial, antioxidant activity and cytotoxicity of the secondary metabolites of *actinomycetes*. The secondary metabolites from the crude extract of Marine *Streptomyces* MS- shows remarkable antibacterial activity against MTCC bacterial pathogens. The antibacterial activity of MS-60 is similar to the results of [33] that gram positive bacteria show good activity than gram negative bacteria. Earlier some marine actinomycetes isolated from Bay of Bengal (Coast of India) were screened for antagonistic and antimicrobial activity against pathogenic bacterial and fungi [34].

Antimicrobial, Antioxidant and Cytotoxic Activity of Marine *Streptomyces* were also studied by Jemimah Naine et al [35]. The concurrent studies of *Streptomyces* sp PM-32 isolated from off-shore sediments collected at the Bay of Bengal coast was reported to have antimicrobial activity against a group of bacterial and fungal pathogens [36]. Zhong [37] et al have recently reported that the EA extract (1 mg/ml) of mycelia of actinomycetes, *Streptomyces* strain Eri12 isolated from rhizosphere of *Rhizoma curcumae Longae* and collected from the Ya'an city of Sichuan province, Southwest of China, showed DPPH• radical scavenging activity (51.87%). Saurav [38] et al studies show that the compound isolated from marine actinomycetes *Streptomyces* sp VITSVK5 has been shown to exhibit 59.32% scavenging activity against DPPH• of free radical at the concentration of 10 µg/ml. The *in vitro* cytotoxicity of secondary metabolites showed a significant antiproliferative activity on HT-29 (Human colon cancer cell line and a dose dependent effect was observed (IC₅₀ – 26.5µg/ml). Some bioactive compounds were isolated and found selectively cytotoxic against lung and colon cancer cell lines as well as melanoma. Interestingly, the compound exerts preferential antiproliferative effects in colon cancer cell lines with defective p53 systems [39]. Matz et al [40] studies of the biological activity compounds revealed that a few strains had anticancer activity: *Eb6*, with an IC₅₀ value of 2.8 µg/ml; *Cc1 4*, with an IC₅₀ value of 6.4 µg/ml. Hellio et al [41] anticancer studies showed the compound of *Sm6*, have an IC₅₀ value of 5.5 µg/ml against colon cancer cells (HCT-116).

5. Conclusion

Marine organisms produce several active chemicals such as antioxidant and antimicrobial compounds. The present study

also indicated that among the marine actinomycete isolates, *Streptomyces* is the dominant genera and revealed that the diversity of marine actinomycetes from Bay of Bengal and their potential as a source of novel bioactive compounds. It remains important to discover new leader compounds from *Streptomyces* for drug development. In this context, the present study highlighted the antimicrobial, antioxidant and cytotoxic potential of crude extract of a Marine *Streptomyces* species MS-60 isolated from the Bay of Bengal, Tamilnadu, India. The Marine *Streptomyces* species shows remarkable activity for both gram positive and gram negative organisms and its antioxidant activity IC₅₀ 12 µg/ml.

The crude metabolite from *Streptomyces* spp. had inhibitory action against the growth of Human colon cancer cell line [HT-29]. The *in vitro* cytotoxicity assay on revealed that the secondary metabolite had the strongest cytotoxicity with IC₅₀ 26.50µg/ml. The bioactive compounds from Marine *streptomyces*. is less toxic to cells. It could be considered as a potential source for anti-cancer drug development. This study suggests that marine-derived actinomycetes are worthy of further exploration as novel drug candidates.

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