Cytotoxic and antimicrobial activities of microbial proteins from mangrove soil actinomycetes of Mangalore, Dakshina Kannada

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ABSTRACT

Introduction and Aim: Actinomycetes are Gram-positive microbes that share characters of both bacteria and fungi, which are distributed uniformly across the world. The current work was focused on identification of bioactive protein compounds from soil actinomycetes isolated from mangrove region of Mangalore, Dakshina Kannada, Karnataka, India.

Materials and Methods: The mangrove soil was subjected to preheat treatment; serial dilution of 10^{-7} was used for plating technique in triplicates. After 7 days of incubation, pure culture isolates were selected and maintained in Yeast Extract Agar (YEA) media. The isolates were examined for colony characteristics and cultivated on YEA media for detection of major metabolites including specific proteins. These extractions were tested for antimicrobial activity by well diffusion technique. Cytotoxicity assay was performed upon physical (UV) and chemical (5% H_2O_2) mutagen treated proliferative yeast cells.

Results: In the present study, the selected mangrove habitat has proved a good source for isolating diversified actinomycetes cultures. These actinomycetes cultures served as the best source for microbial proteins having a biological activity. Protein extraction by ammonium sulfate precipitation and dialysis yielded protein components that showed antimicrobial activity against Gram positive and Gram-negative bacteria in comparison to standard streptomycin. The protein components that showed the highest antimicrobial activity were selected and preserved as antimicrobial peptides (AMPs). Further, these proteins also showed effective cytotoxic activity against proliferative yeast cells.

Conclusion: Mangrove actinomycete isolates proved as one of the best natural sources for isolation of novel compounds. Specific proteins that could target antimicrobial activity as well as mechanism of programmed cell death in both cancerous and endothelial cells were initialized using yeast cells by performing cytotoxic activity against proliferative yeast cells.

Keywords: Mangrove; actinomycetes; microbial protein; antimicrobial; cytotoxicity.

INTRODUCTION

Actinomycetes are a major group of microbial population requiring scientific investigations and research findings for identification of novel compounds which are useful for social well-being. They have exceptional and unusual ability for surviving in diverse habitats and are widely distributed across the world in different habitats, a natural and everlasting source for production of commercially useful enzymes and therapeutically bioactive molecules. They produce a number of enzymes that help degrade organic plant material, lignin and chitin. Their presence is important in the formation of compost. Certain species are commensal in skin flora, oral flora, gut flora, and vaginal flora of humans and livestock. Due to their ability to survive in conditions of stress and adapt to the environment, there could be a chance for isolation of novel strains (1).

Actinomycetes are aerobic, spore forming and Gram-positive bacteria. It belongs to order Actinomycetales characterized by the growth of substrate and aerial mycelium. It is one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria. The "Actinomycetes" word is derived from Greek "atkis" (a ray) and "mykes" (fungus), shows characteristics of both bacteria and fungi but possesses sufficient distinctive features to delimit them into 'Kingdom bacteria'. The actinomycetes are potential producers of antibiotics and other therapeutically useful compounds (2).

Actinomycetes are known to be the most prolific producers of biologically active metabolites. These compounds often show potent biological activity and thus have been recognized for their pharmaceutical and biotechnological potential. Further, these compounds are influenced by the physiological environment at the

geographical location (3). The bioactive secondary metabolites produced by actinomycetes include antibiotics, immunosuppressive agents, antitumor agents and enzymes. These metabolites are known to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti-inflammatory activity. Actinomycetes have proved their ability to produce a variety of bioactive secondary metabolites and for this reason, the discovery of novel antibiotic and non-antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important (4).

Mangrove habitat in Mangalore region of Dakshina Kannada is a unique ecosystem having high salinity, high temperature, extreme tides, high sedimentation and high evaporation. The muddy anaerobic mangrove differs from the terrestrial microbial diversity. These are largely unexplored and offers excellent opportunity for finding novel actinomycetes with unique properties. The Karnataka state has a sizeable stretch of mangrove forests, a vibrant saline-water ecosystem associated with India's coastal region (5).

The microbial community inhabiting this niche has not been well characterized. The diversity of the actinomycete population was studied and evaluated based on biochemical and morphological characteristics to evaluate the biosynthetic potential of these isolates. The profiles obtained for each isolate were compared to the antimicrobial activity exhibited by isolates in laboratory conditions. The results obtained from the isolates were positive, as producers of metabolites with biological activities as little is known about the diversity of the microbial community inhabiting these environments (6).

MATERIALS AND METHODS

Soil sample collection

Mangrove soil was collected from four different places in the region of Mangalore, Karnataka, India. The collected samples were transported aseptically in sterile plastic bags to the Molecular Research Laboratory, Department of Microbiology, Jnana Kaveri, Mangalore University. The samples were subjected to preheat treatment at 60˚C for 2 hours to avoid bacterial and fungal growth before serial dilution and plating technique. The texture of soil samples varied from sandy to loamy and parameters such as temperature showed 21˚C at the site of collection and pH 7.2 was recorded.

Isolation of actinomycetes

One gram of soil sample was serially diluted up to 10^{-7} dilution. Aliquots of 0.1ml of each dilution was spread, plated on Yeast Extract Agar (YEA) plates in triplicates and incubated at 30˚C for 7 days. After incubation time, the plates were examined for presence of actinomycetes colonies. The prominent colonies were selected, and pure cultures were maintained on YEA media.

Culture characteristics

Each of the actinomycetes colonies was analyzed for morphological characteristics such as substrate and aerial mycelia, spore bearing hyphae with spore pattern using *LYNX* Inverted Phase Contrast Microscope.

Growth on suitable media

To check for the efficient growth of each actinomycete isolates, four different media were used for isolation such as Starch Casein Agar (SCA), Yeast Extract Agar (YEA), Yeast Malt Extract Agar (YMA), Kustar's Agar (KA), and Isolation Agar (IA). The colony growth on SCA and YEA allowed for the highest recovery and best suited for isolation. SCA media composition (Starch: 1%, Casein: 0.03%, KNO_3: 0.2%, $MgSO_4.7H_2O$: 0.005%, K_2HPO_4: 0.2%, NaCl: 0.2%, $CaCO_3$: 0.02%, $FeSO_4$: 0.001%, Agar: 2%) and YEA media composition (D-Mannitol: 1%, K_2HPO_4: 0.05%, $MgSO_4.7H_2O$: 0.2%, NaCl: 0.1%, $CaCO_3$: 0.38%, Yeast Extract: 0.1%, Congo red solution: 0.00025%, Agar: 2%) were prepared (7). All chemicals were procured from HiMedia, India.

Protein extraction

Mangrove actinomycetes isolates were tested for antibacterial activity. The isolates of actinomycetes were inoculated into YE broth and incubated at 30˚C under shaker condition set at 100 rpm for 7 days. The broth culture was centrifuged at 10000 rpm at 4˚C for 15 min to obtain culture pellet that was subjected for protein isolation. The biomass was homogenized with 0.1M phosphate buffer pH 7.2 and centrifuged which yielded crude protein extract. The extract was detected and estimated for the presence of protein using Lowry's method. Further, salt precipitation using ammonium sulfate (80% saturation) and dialysis for salting out yielded partially purified microbial protein.

Antimicrobial activity

Around 20µg of partially purified microbial protein was used for antimicrobial activity by well diffusion method (8). Pathogenic test organisms selected for the study were *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC9621), *Staphylococcus aureus* (ATCC6538P). Nutrient agar plates were prepared, and swab inoculation of the pathogens was made on the surface to produce a lawn culture. 20µg of partially

purified protein were loaded on to well of inoculated agar with a diameter of 6.0mm. The plates were incubated at 30°C for 24 hours and inhibitions were observed as zone of clearance. Zone of inhibition was measured and recorded (9).

Testing of cytotoxicity

The unicellular eukaryotic *Saccharomyces cerevisiae (*24 hours old) was maintained as stock culture. The yeast cells were inoculated separately in fresh Yeast Extract Malt broth. After growth for 24 hours, yeast culture broth with constant proliferative rate were separated and maintained as control, 5%H_2O_2 treatment (chemical mutagen) and exposure to ultraviolet rays for 1hour (physical mutagen). Cell counting was performed using Neubauer's chamber after staining with 0.4% trypan blue. At 0 hour all the three broth cultures showed same cell count, after treatment with chemical and physical mutagen proliferative rate of yeast cell increased and maintained as cell line to test the efficacy of actinomycetes crude protein extract. About 20µg of protein was tested in yeast culture broth (1ml) against the chemical and physical mutagen treated yeast cells (10-12).

RESULTS

Isolation of mangrove actinomycetes

Prominent actinomycetes were selected based on their efficient growth on YEA media. Preheat treatment of mangrove soil sample reduced the bacterial and fungal growth resulting in the isolation of potential actinomycete candidates. Based on colony, morphological and biochemical characteristics, around 46 actinomycetes isolates were sub-cultured and maintained in the Molecular Research Laboratory, Department of Microbiology, Jnana Kaveri of Mangalore University. The mycelium and spore arrangement pattern were analyzed using inverted phase contrast microscope and documented which are shown in table 1.

Table 1: Morphological characteristics of actinomycetes grown on Yeast Extract Agar (YEA)

Mangrove Actinomycetes Isolate	Texture	Pigmentation	Color of aerial mycelia	Color of substratum	Gram staining	Colony features	
Y1	Powdery	+ve	White	Black	+ve	Elevated	Circular
Y2	Slimy	+ve	Pale creamish	Colorless	+ve	Flat	Circular
Y3	Powdery	+ve	Black	Black	+ve	Elevated	Irregular
Y4	Powdery	+ve	Orange	Red	+ve	Flat	Circular
Y5	Slimy	+ve	Pinkish	Colorless	+ve	Elevated	Irregular
Y6	Slimy	+ve	White ash	Pink	+ve	Elevated	Circular
Y7	Powdery	+ve	White	Black	+ve	Elevated	Irregular
Y8	Slimy	+ve	Pale Orange	Colorless	+ve	Elevated	Circular
Y9	Slimy	+ve	Orange	Red	+ve	Elevated	Circular
Y10	Powdery	+ve	White	Black	+ve	Flat	Circular
Y11	Slimy	+ve	Pinkish	Colorless	+ve	Elevated	Irregular
Y12	Slimy	+ve	Pale Orange	Red	+ve	Elevated	Circular
Y13	Powdery	+ve	Orange	Red	+ve	Flat	Circular
Y14	Powdery	+ve	Light Creamish ash	Pink	+ve	Elevated	Circular
Y15	Slimy	+ve	Pinkish	Light Pink	+ve	Elevated	Irregular
Y16	Powdery	+ve	White	Colorless	+ve	Elevated	Irregular
Y17	Slimy	+ve	Pale Orange	Red	+ve	Elevated	Circular
Y18	Powdery	+ve	Pale yellow	Brown	+ve	Elevated	Circular
Y19	Slimy	+ve	White	Colorless	+ve	Elevated	Circular
Y20	Powdery	+ve	Red	Black	+ve	Elevated	Circular
Y21	Powdery	+ve	Pink	White	+ve	Elevated	Circular
Y22	Powdery	+ve	White	Black	+ve	Flat	Irregular

Y23	Powdery	+ve	White	Pink	+ve	Flat	Circular
Y24	Powdery	+ve	Orange	Black	+ve	Elevated	Irregular
Y25	Slimy	+ve	Orange	Red	+ve	Elevated	Circular
Y26	Slimy	+ve	Red	Colorless	+ve	Elevated	Circular
Y27	Powdery	+ve	Orange	Black	+ve	Elevated	Circular
Y28	Slimy	+ve	Creamish	Colorless	+ve	Elevated	Circular
Y29	Slimy	+ve	Pinkish	Colorless	+ve	Elevated	Circular
Y30	Powdery	+ve	Light Pink	Colorless	+ve	Elevated	Irregular
Y31	Powdery	+ve	Pink	White	+ve	Elevated	Circular
Y32	Slimy	+ve	Pink	Colorless	+ve	Elevated	Circular
Y33	Slimy	+ve	Yellow ash	Brown	+ve	Elevated	Circular
Y34	Slimy	+ve	Creamish	Colorless	+ve	Elevated	Circular
Y35	Slimy	+ve	Creamish	Colorless	+ve	Elevated	Circular
Y36	Powdery	+ve	Creamish	Colorless	+ve	Elevated	Irregular
Y37	Slimy	+ve	Black Ash	Red	+ve	Elevated	Circular
Y38	Slimy	+ve	Black	Pink	+ve	Elevated	Circular
Y39	Slimy	+ve	Black	Red	+ve	Elevated	Circular
Y40	Slimy	+ve	White	Colorless	+ve	Elevated	Irregular
Y41	Powdery	+ve	Yellow ash	Red	+ve	Elevated	Circular
Y42	Powdery	+ve	Orange	Red	+ve	Elevated	Circular
Y43	Slimy	+ve	Pink	Colorless	+ve	Elevated	Circular
Y44	Powdery	+ve	White	Colorless	+ve	Elevated	Circular
Y45	Powdery	+ve	Orange	Orange	+ve	Elevated	Irregular
Y46	Powdery	+ve	Red	Pink	+ve	Elevated	Circular

Extraction of protein

The cell biomass and the cell free culture extracts from each of the isolates were obtained after their growth on YE broth grown up to 7 days. The cell biomass after homogenization with 0.1M phosphate buffer resulted in the detection of protein and protein yield was significant for further assays. All the selected isolates showed more than 10mg protein/1g of cell biomass.

Antibacterial activity

The protein samples obtained from each isolate were tested for antimicrobial activity against *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 9621) *Staphylococcus aureus* (ATCC 6538P) and result obtained is shown in table. 2. When protein samples extracted from all forty-six actinomycetes cultures were tested, fourteen protein samples showed effective antibacterial activity. The zone of inhibition was recorded with reference to standard antibiotic streptomycin (10μg) that showed 10mm inhibition activity against test pathogens. This result indicates that protein obtained from the cell biomass contained anti-microbial peptides (AMPs) that could be novel with respect to the habitat and mechanism of antimicrobial action.

Table 2: Antimicrobial activity of actinomycetes protein (AMP) extract from Y1 to Y46

Actinomycete Protein Sample	Zone of Inhibition (mm)			
	E. coli	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
Y1	-	7.0	-	-
Y2	6.0	6.0	-	-
Y3	8.0	-	6.0	6.0
Y4	6.0	6.0	-	-
Y5	-	7.0	6.0	7.0
Y6	-	6.0	6.0	6.0
Y7	7.0	-	8.0	-
Y8	8.0	6.0	-	6.0
Y9	6.0	6.0	7.0	11.0
Y10	8.0	6.0	6.0	7.0

Y11	6.0	6.0	-	7.0
Y12	-	-	-	13.0
Y13	-	-	-	-
Y14	-	8.0	-	-
Y15	7.0	8.0	-	6.0
Y16	6.0	8.0	-	-
Y17	12.0	9.0	6.0	-
Y18	16.0	7.0	-	-
Y19	12.0	7.0	7.0	-
Y20	10.0	6.0	-	8.0
Y21	6.0	6.0	8.0	-
Y22	7.0	6.0	-	-
Y23	-	8.0	-	6.0
Y24	-	6.0	-	-
Y25	6.0	6.0	-	6.0
Y26	8.0	-	6.0	6.0
Y27	6.0	6.0	-	-
Y28	6.0	6.0	-	6.0
Y29	7.0	-	-	-
Y30	7.0	6.0	-	-
Y31	8.0	-	-	7.0
Y32	6.0	8.0	-	7.0
Y33	-	6.0	-	7.0
Y34	-	6.0	-	6.0
Y35	-	7.0	-	-
Y36	-	10.0	14.0	7.0
Y37	6.0	6.0	15.0	-
Y38	-	7.0	6.0	-
Y39	-	7.0	17.0	-
Y40	6.0	8.0	15.0	-
Y41	6.0	6.0	14.0	6.0
Y42	-	-	13.0	8.0
Y43	-	6.0	-	6.0
Y44	6.0	-	-	6.0
Y45	7.0	15.0	16.0	7.0
Y46	6.0	13.0	8.0	8.0

Cytotoxic activity

The protein from each of the isolates when assayed for cytotoxicity against normal yeast cellular proliferation and abnormal (Chemical and Physical) mutagen treated yeast cells showed significant cytotoxicity against highly proliferative yeast cells. Comparing the result of cytotoxicity with respect to 5% H_2O_2 and UV radiated yeast cell, upon treatment with protein extracts the isolates such as isolates Y3, Y6, Y14, Y15, Y18, Y33, Y37, Y38, Y39, Y41, Y45, Y46 have shown significant cytotoxicity against chemical and physical mutagen treated yeast cells as shown in graph 1 and graph 2. This result thus attributes towards the identification of anticancer peptides (ACP's). As shown in Fig. 1, the growth pattern of the selected actinomycete isolates were analyzed on YEA media. All the selected isolates have shown varied pigmentation and are morphologically diversified. All have shown Gram staining positive.

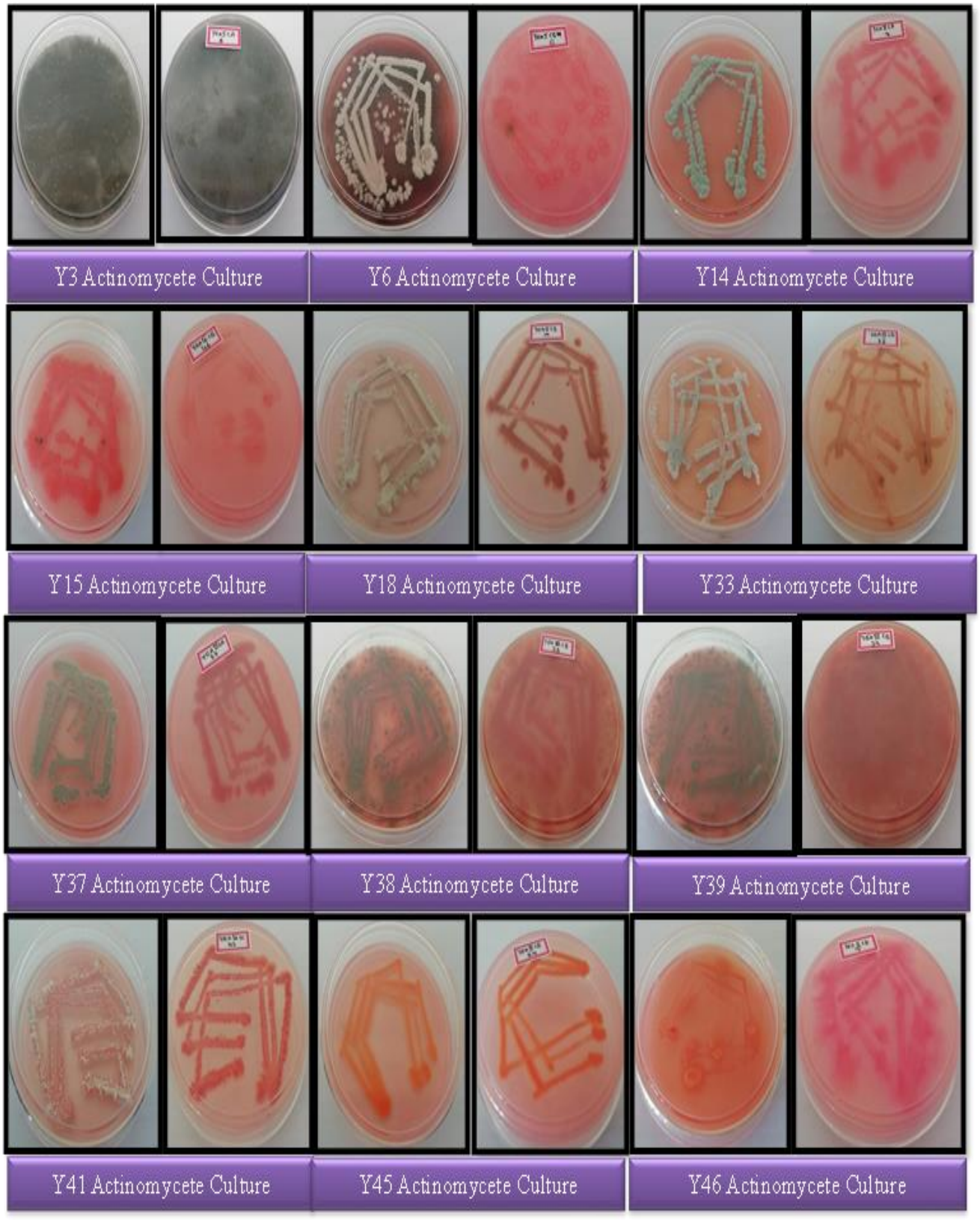
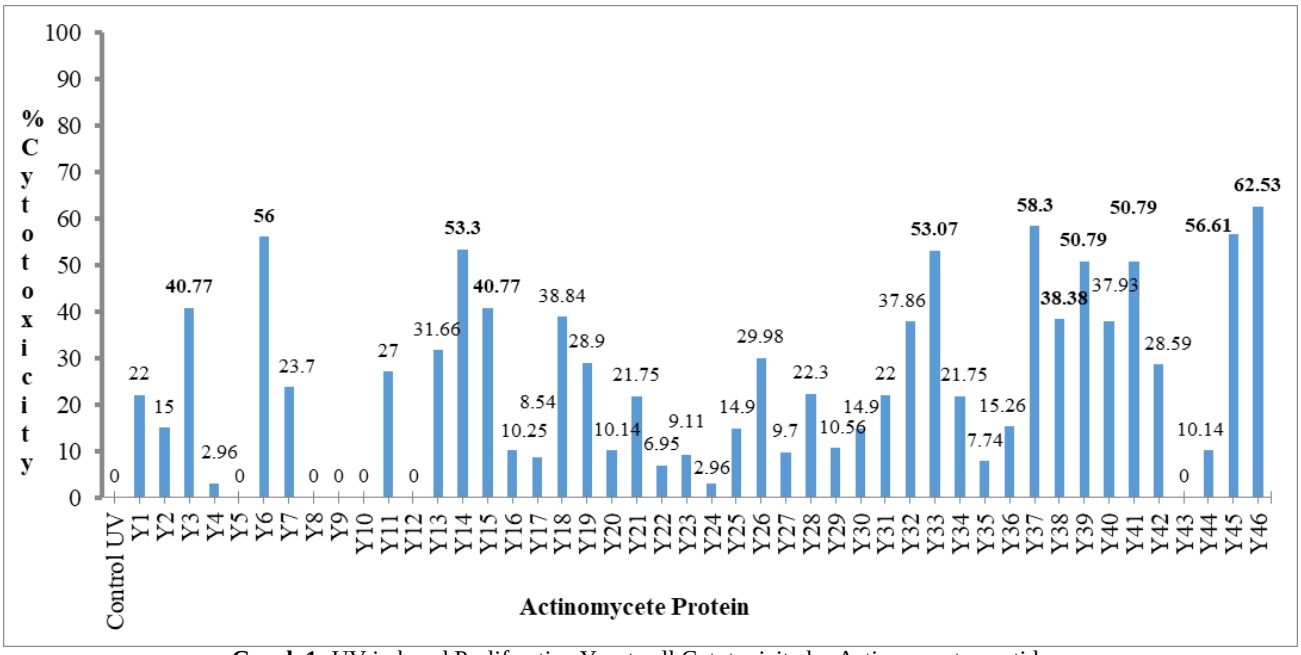
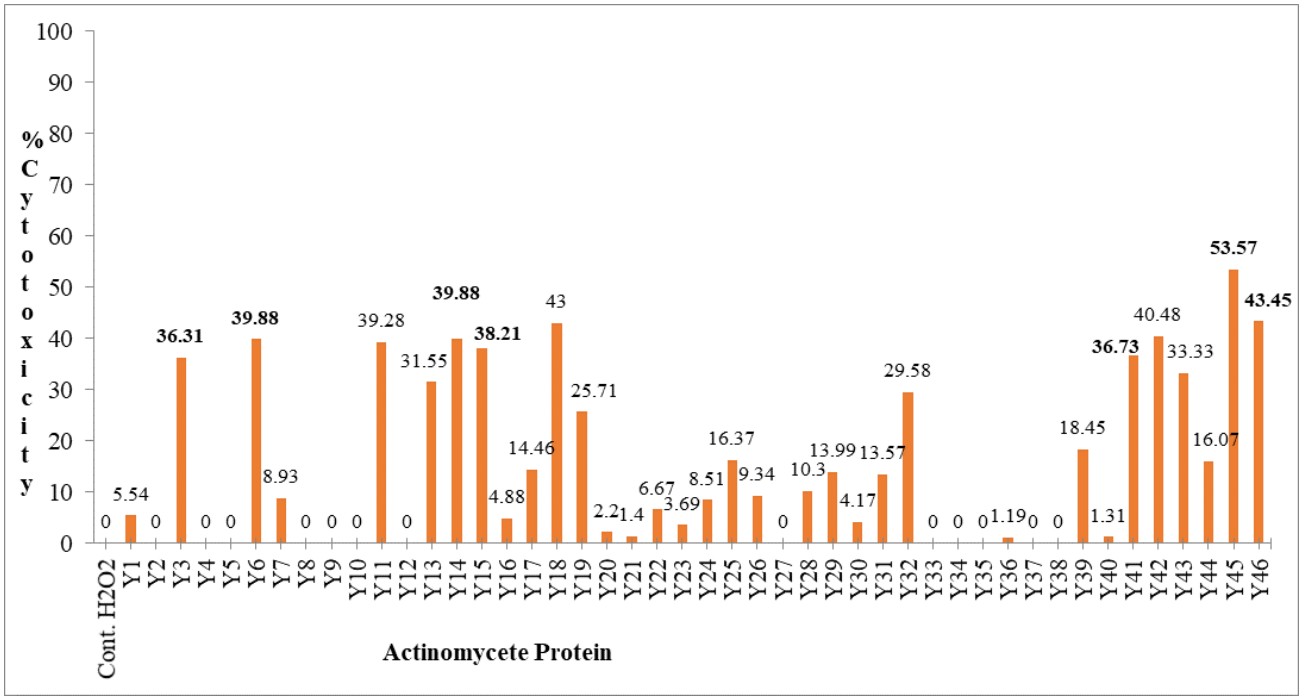


Fig 1. Potential Mangrove Actinomycete culture grown on Yeast Extract Agar (YEA) media



Graph 1: UV induced Proliferative Yeast cell Cytotoxicity by Actinomycete peptides.



Graph 2: H_2O_2 induced proliferative yeast cell cytotoxicity by actinomycete peptides

The cytotoxicity results of the protein extract from above isolates when compared with antimicrobial activity leads to the identification of antimicrobial peptides (AMPs) also having anticancer properties as shown in table 3. This result gives insight understanding about the protein activities that are antimicrobial will also act as anticancer peptides.

Table 3: Comparison of percentage cytotoxicity and antimicrobial activity of protein extracts of isolates

Actinomycetes Isolates	Percentage Cytotoxicity		Antimicrobial Activity
	UV induced Proliferation	5%H_2O_2 induced Proliferation	
Y3	40.77	36.33	+++
Y6	56.0	39.88	+++
Y7	23.7	8.93	++
Y11	27.0	39.28	+++

Y14	53.3	39.88	+
Y15	40.77	38.21	+++
Y18	38.84	43.0	++
Y19	28.9	25.71	+++
Y25	14.9	16.37	+++
Y32	37.86	29.58	+++
Y37	58.3	-	+++
Y38	38.8	-	++
Y39	50.79	18.45	++
Y41	50.79	36.73	++++
Y42	28.59	40.48	++
Y45	56.6	53.57	++++
Y46	62.53	43.43	++++

++++ (shown activity against four test organisms)
+++ (shown activity against three test organisms)
++ (shown activity against two test organisms)

DISCUSSION

Exploration of microorganisms for their inherent physiological and functional diversity is widely applied in medicine, agriculture, industry and environmental research. Among diverse industrially used microorganisms, actinomycetes are important and predominantly used in antibiotic studies. Actinomycetes are Gram-positive filamentous bacteria having high GC content and potential producer of secondary metabolites (13). Recent studies are in search of novel compounds from the well-known actinomycetes strains isolated from different habitats (14). The limited research data available on actinomycetes population across the world and a very few reports are available from India. Therefore, unexplored areas and unusual habitats offer good source for new compounds.

During the search of target specific mechanism of antimicrobial and anticancer activity, mangrove actinomycetes could be considered as one of the best sources for exploring novel compounds. Mangrove ecosystem of coastal Karnataka is still unexplored for unprecedented potential active compounds. This habitat is unique from other habitats in terms of high salinity, rich in sulphide concentration with organic matter. Hence, in order to search novel compounds, we isolated and characterized bioactive proteins from mangrove soil actinomycetes of Mangalore, Dakshina Kannada. These isolated bioactive proteins were tested against *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumoniae* (ATCC 9621) *Staphylococcus aureus* (ATCC 6538P) and their antimicrobial activities were determined.

It is reported that antimicrobial peptides are cationic and have low molecular weight and specifically target anionic cell membrane. This property of antimicrobial peptides is speculated to have antimicrobial activity by targeting bacterial cell membrane. Further, these antimicrobial peptides offer as potential compounds even to target against anionic surface markers on cancerous cell membrane thus disrupting cancer cell growth.

The bioactive peptides in the present work considered as antimicrobial peptides which were checked for cytotoxic activity against *Saccharomyces cerevisiae*. According to Bjornsti *et al.*, *Saccharomyces cerevisiae* served as a eukaryotic cell model that shared genetic homology with human genetic system (15). In the present study, yeast cell cytotoxicity testing was initiated with the obtained antimicrobial peptides, after yeast cells were transformed by treating with physical and chemical mutagens. The present work substantiates to determine antimicrobial peptides that also serve as anticancer peptides. Therefore, around eighteen isolates have shown antimicrobial and cytotoxic activity by the respective peptides. The protein compound from actinomycete isolates Y3, Y6, Y11, Y15, Y19 have shown significant antimicrobial effect against three test organisms and also more than 25% of cytotoxic activity. However, actinomycete isolate Y41, Y45, Y46, have shown significant antimicrobial effect against four test organisms and with more than 50% cytotoxic activity. Therefore, these three actinomycete isolates are thus beneficial for exploring novel peptides with target specific activity.

CONCLUSION

In the process of exploration of potential microorganisms, the actinomycetes in the present study have shown positive results in the production of antimicrobial peptides that have proven cytotoxicity effect and therefore can be considered as effective anticancer peptides. Three of the isolates, actinomycetes isolate Y41, Y45, Y46, have shown significant cytotoxicity as well as antimicrobial

property. Mangrove actinomycetes are the best natural sources for production of primary and secondary metabolites which are efficient for various therapeutic applications. Hence, actinomycetes isolates from mangrove habitat have served as the best sources for isolation of new bioactive protein compounds for induction of programmed cell death in cancerous and pathological angiogenic endothelial cells thus attributing towards target specific activity apart from various biological activities.

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REFERENCES

1. Steinert, G., Taylor, M. W., Schupp, P. J. Diversity of actinobacteria associated with the marine ascidian eudistomatoealensis. Mar. Biotechnol. 2015; 17: 377- 385.
2. Embley, T. M., and E. Stackebrandt. The molecular phylogeny and systematics of the actinomycetes. Annu. Rev. Microbiol. 1994; 48: 257- 289.
3. Hirsch, P., Mevs, U., Kroppenstedt, R. M., Schumann, P. and Stackebrandt, E. Crytpo endolithic actinomycetes from antarctic sandstone: *Micromonospora endolithica* sp. nov. and two isolates related to *Micromonospora coerulea* Jensen 1932. Syst. Appl. Microbiol. 2004; 27: 166- 174.
4. Hoskin, D. W., and Ramamoorthy, A. Studies on anticancer activities of antimicrobial peptides. Biochim. Biophys. Acta. 2008; 1778: 357- 375.
5. Pariona, Ameber. (2017, August 1). What Is A Mangrove Habitat?Retrieved from https://www. worldatlas.com/ articles/ what-is-a-mangrove-habitat.html
6. van Zoggel, H., Carpentier, G., Dos Santos, C., Hamma-Kourbali, Y., Courty, J., Amiche, M., *et al.* Antitumor and angiostatic activities of the antimicrobial peptide dermaseptin B2. 2012; PLoS ONE 7: e44351.
7. Nayana and Manjula, I. K: Cysteine Protease Inhibition and Anti-Proliferative Activity of the Protein Fraction Isolated from *Justicia wynaadensis*, J. Pharm. Res. 2015; 4(4): 167-171.
8. Rajeshwari, K., Manjula, I. K. Inhibition of Cysteine proteases and antibacterial activity of Rhizosphere-microbial isolates of J*usticia wynaadensis*, Int J pharm Bio Sci. 2015 July; 6(3): (B) 131- 138.
9. Sengupta S., Pramanik A., Ghosh, A., Bhattacharyya, M. Antimicrobial activities of actinomycetes isolated from unexplored regions of Sundarbans mangrove ecosystem. BMC Microbiology. 2015; 15: 170.
10. Ashwini, B. and Manjula, I. K. Protein fraction from actinomycetes isolates inhibits protease activity secreted by *staphylococcus aureus,* European journal of pharmaceutical and medical sciences, 2015; 2(7): 186- 193.
11. Manjula, I. K., Sheela, M. L. & Bharathi, P. Salimath. A pro-apoptotic 15 k Da protein from *Bacopa monnieri* activates caspase-3 and down regulates bcl-2 gene expression in mouse mammary carcinoma cells. J Nat Med. 2013; 67 (1): 123- 136.
12. Sathiyaseelan K., Stella, D. Isolation, Identification and antagonistic activity of marine actinomycetes isolated from the Muthupet mangrove environment. International Journal of Pharmaceutical and Biological Archives. 2011; 2(5): 1464-1468.
13. Hu, D., Chen, Y., Sun, C., Jin, T., Fan, G., Liao, Q., *et al.* Genome guided investigation of antibiotics producing actinomycetales strain isolated from a Macau mangrove ecosystem. Scientific reports. 2018; 8(1): pp.1-12.
14. Gomez-Escribano, J. P., Alt, S. and Bibb, M. J. Next generation sequencing of actinobacteria for the discovery of novel natural products. *Marine drugs*. 2016; *14*(4): p.78.
15. Bjornsti, M. A., Knab, A. M. and Benedetti, P. Yeast Saccharomyces cerevisiae as a model system to study the cytotoxic activity of the antitumor drug camptothecin. Cancer Chemotherapy and Pharmacology. 1994; *34*(1): pp. S1-S5.