BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2015; 10: 57-62
ISSN: 1991-0088

In vitro cytotoxic and antibacterial potentials of extracts from three marine isolates of Actinomycetes isolated from coastal ecosystems of Tanur, Kerala, India

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Article Info

Received: 2 December 2014
Accepted: 29 December 2014
Available Online: 16 January 2015

DOI: 10.3329/bjp.v10i1.21085

Cite this article:
Prashanthi K, Varalakshmi KN. *In vitro* cytotoxic and antibacterial potentials of extracts from three marine isolates of Actinomycetes isolated from coastal ecosystems of Tanur, Kerala, India. Bangladesh J Pharmacol. 2015; 10: 57-62.

Abstract

Three Actinomyctes with potential bioactivity are successfully isolated from the marine water samples and identified as *Prauserella marina, Streptomyces sindenensis* and *S. spiroverticillatus*. The ethyl acetate extracts from the three Actinomycetes are found to have significant bioactivity. The highest antibacterial activity was given by the extract from *P. marina* on *B. cereus* showing 28 mm of zone of inhibition. Cytotoxicity screening of the crude extracts using 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell viability assay revealed that extract from *P. marina* noticeably effected the viability of the human cervical cancer cell grown *in vitro*. Thin layer chromatography of the crude extract with methanol and chloroform (8:2) as solvent system yielded three distinct fractions, of which fraction with Rf value 0.8 resulted in 77, 68, 54 and 40% growth inhibition of HeLa cells at 15, 10, 5, 2.5 µg/mL, respectively with the IC_{50} value as 3.3 µg/mL. HPLC analysis of the fraction resulted in single major peak at 3.7 min.

Introduction

Actinomycetes are Gram-positive filamentous organisms best known as major source of antibiotics. They belong to the order Actinomycetales (Super-kingdom: Bacteria, Phylum: Firmicutes, Class: Actino-bacteria) (Ventura et al., 2007). Among Actinomycetes, majority of the antibiotics are produced by *Streptomyces* species. Members of this group of bacteria are the producers of vast variety of bioactive compounds with applications in several fields (Berdy, 2005). Despite the advancement in the knowledge of cancer induction and progression, cancer still remains one of the most fatal illnesses affecting human health (Olano et al., 2009). Chemotherapy is one of the most preferred treatments of choice for cancer. Many clinically useful antitumor drugs such as anthracyclines (aclarubicin, daunomycin and doxorubicin), peptides (bleomycin and actinomycin D), aureolic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins and others are produced by Actinomycetes. With the rapid development of resistance to multiple chemotherapeutic agents, high toxicity to drugs and a significant number of side effects, there is a need to search for novel chemotherapeutic agents (Newman and Cragg, 2007).

Marine Actinomycetes are widely distributed in different marine ecosystems and are largely unexplored. To ensure survival in environments like extreme pressure, temperature and pH, marine Actinomycetes have acquired unique metabolic and physiological capabilities (Haefner, 2003; Bull and Stach, 2007). As there is difference in the characteristics of marine Actinomycetes from their terrestrial counterparts, the secondary metabolites from marine source may be promising as bioactive compounds (Stach et al., 2003; Jensen et al., 2005). The present study is an attempt to explore the possibilities of finding a novel chemotherapeutic agent against cancer and bacterial human pathogens from

three Actinomycetes isolated from costal marine ecosystem.

Materials and Methods

Collection of water sample

Marine water samples for the current study were collected from different locations around Tanur beach, Malappuram district, Kerala, India (10.97N 75.86E), in sterile containers and were stored at 4°C.

Isolation and morphological characterization

Isolation was carried out by serial dilution and spread plate technique (Kumar et al., 2010). The dilutions were made in sterilized marine water up to 10^{-5} dilutions and were spread on Starch Casein Nitrate Agar (SCNA) medium with tetracycline and fluconazole added to inhibit the growth of bacteria and fungi respectively. The cultures were incubated for 7 days at 28°C. Pure cultures were obtained by subculturing on slants containing SCNA and maintained at 4°C for further study. Actinomycetes were identified by comparing their colony morphology (Oskay et al., 2005), spore-bearing hyphae and structure of spore chain with the Actinomycetes morphologies, as described elsewhere (Cross 1989; Goodfellow 1989).

Molecular characterization and phylogenetic analysis

Actinomycete isolates were cultured in SCN broth and incubated for 5 days at 28°C at 150 rpm. The cell mass was filtered and used for genomic DNA isolation. DNA extraction was carried out using genomic DNA isolation kit (Chromus Biotech Pvt. Ltd., Bangalore, India). Specific sequence amplification was performed using universal 16S rDNA primers (Schwieger and Tebbe 1998). The reaction was set in a total 25 µL reaction mixture in sterile 0.2 mL PCR vials. The reaction mixture contained the following components: 1.5 µL of genomic DNA, 10 picomoles of 16S rDNA forward primer (5′- AGAGTTTGATCCTGGCTCA – 3′), 10 picomoles of 16S rDNA reverse primer (5′- ACGGC-TACCTTGTTACGACT – 3′), 1 µL of 30 mM deoxyribo-nucleoside 5′ triphosphate (all the dNTPs in equimolar concentration), 2.5 µL of 10X PCR assay buffer and 1 µL of Taq DNA polymerase enzyme (1U) (Chromus Biotech Pvt. Ltd., Bangalore, Karnataka, India). Sterile distilled water was added to make up the volume to 25 µL. The vials were then set in thermal cycler (MJ research, PTC 200) for amplification with the following program: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation – 1 min at 94°C, annealing - 30 sec at 57°C and extension - 1 min at 74°C. The program ended with final extension step - 5 min at 74°C. The amplified product was analyzed on 1% agarose gel and documented using gel documentation system (Herolabs, Germany). The amplified PCR product was then sequenced at Chromus Biotech Pvt. Ltd., Bangalore, Karnataka, India, for obtaining partial sequence. The sequences were analyzed using Molecular Evolutionary Genetics Analysis software (MEGA, version 6.05) (Tamura et al., 2011) and aligned together with reference sequences obtained from the GeneBank, EMBL and DDBJ using the CLUSTAL W program included in MEGA 6.05. Phylogenetic trees were constructed using the software MEGA 6.05 based on the neighbor-joining method (Saitou and Nei, 1987; Tamura et al., 2011). The topology was built by the bootstrap method (1,000 replicates) (Felsenstein, 1985).

Ethyl acetate extraction

The antagonist *Actinomycetes* were inoculated in 50 mL yeast extract malt extract (Shirling and Gottlieb, 1966) broth and incubated at 28°C in a shaker incubator at 150 rpm for 10 days. The culture was filtered and to the filtrate equal volume of ethyl acetate was added. The filtrate with ethyl acetate was shaken vigorously for 1 hour. The ethyl acetate fraction was collected using a separating funnel. The solvent was then evaporated, and the extracts were dissolved in DMSO at a concentration of 1 mg/mL for testing the cytotoxicity and antibacterial activity.

TLC fractionation

TLC separation was performed using commercially available silica plates. The solvent mix-ture for the TLC fractionation was optimized by trial and error method. For each trial, a 1 cm x 6 cm silica plate was used and the sample was spotted using a thin capillary tube just 1 cm above the bottom of the plate. The solvent systems with chloroform, methanol, ethyl acetate and water were tested with different combina-tions. The silica plate was kept in a closed chamber with the mobile phase and run till the solvent reached the top end of the plate. The plate was then dried, and the spots were detected by exposing the plate to UV light and iodine vapors. The distance of each spot from the origin was measured and Rf values were calculated. For the preparative TLC, the fractions were scraped care-fully from the TLC plates and dissolved in methanol. The silica particles were separated by centrifugation, and each fraction was tested for cytotoxic and antibacterial activity (Kirchner et al., 1951).

Cytotoxicity Testing - MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] cell viability assay

HeLa cells were seeded in 96-well flat-bottom microplates and cultured in a CO_2 incubator (at 37°C with 5% CO_2 and 95% air) for adhesion overnight. The ethyl acetate extracts at 50, 25 and 10 µg/mL and TLC purified frac-tions at 15, 10, 5 and 2.5 µg/mL concentration were added to the ELISA plate , and the plate was incubated for 24 hours, each in

quadruplicates. Following the incubation, 100 µL of MTT solution was added. The culture was then incubated for 3 hours in dark chamber for the conversion of MTT to formazan. Thereafter, the supernatant was aspirated and 100 µL of DMSO was added to dissolve the formazan. The absorbance was recorded at 540 nm with the help of ELISA plate reader (Mosmann, 1983). The percentage viability was calculated using the following formula:

Percentage viability (%) = (A_{540} of the test sample)/ (A_{540} of the control) X 100

Preliminary antimicrobial activity screening

Isolates were preliminarily tested for antimicrobial activity by cross-streak method on SCNA plates (Sateesh et al., 2011). After incubation at 28°C for 6 days, 24-hour cultures of bacteria were streaked perpendicular to the central strip of Actinomycetes culture, the plates were incubated at 30°C for 24 hours and zone of inhibition was noted. The clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *S. citrus*, *Bacillus subtilis*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella*, *Bacillus polymyxa*, *Serratia marcescens* and *Pseudomonas* sp. were used in the study.

Antibacterial activity and zones of inhibitions

Antimicrobial activities of the extracts were tested against different Gram-positive and Gram-negative bacteria using agar well diffusion method (Bauer et al., 1966). Nutrient broth was used for culturing the bacteria. The overnight grown bacterial cultures (100 µL each) were spread on Muller Hilton agar plates. A cork borer with a diameter of 6.0 mm was used for punching the wells on the agar plates. Control and sample extracts (50 µL each) were loaded on to the wells. The inhibition zones around the wells were measured after 24 hours of incubation at 37°C.

HPLC analysis of bioactive fraction

The bioactive fraction from *Prauserella marina* JUACT 04 was analyzed using reverse phase HPLC. The analysis was performed using WATERS HPLC system with binary pump and dual λ absorbance UV detector. The column used was C-18 YMC with 3 µm particle size. The mobile phase consisted of 80:20 of water: methanol (Snyder et al., 2012).

Results

The pure culture colonies of the three Actinomycete isolates were obtained up on serial dilution and were maintained by subculturing on starch casein nitrate agar medium (Figure 1). In the preliminary examination of the morphological and colony characters, the three bacteria isolated from the marine samples were identified as members of the class Actinobacteria. Further molecular characterization using 16 S rDNA analyses resulted in the identification of the organisms as *P. marina* JUACT 04; *Streptomyces sindenensis* JUACT 06; *S. spiroverticillatus* JUACT 05. The phylogenetic analysis revealed that *P. marina* JUACT 04 shows highest affinity with *P. marina strain* Ms498 by 96%, *S. sindenensis* JUACT 06 with *S. sindenensis* strain NBRC 3399 by 89% and *S. spiroverticillatus* JUACT 05 with *S. spiroverticillatus* strain NBRC 12821 by 98% bootstarp level (Figure 2).

The cross streak method of testing was useful in quickly screening the antibiotic properties of the three isolates. It was found that all the three isolates were inhibiting

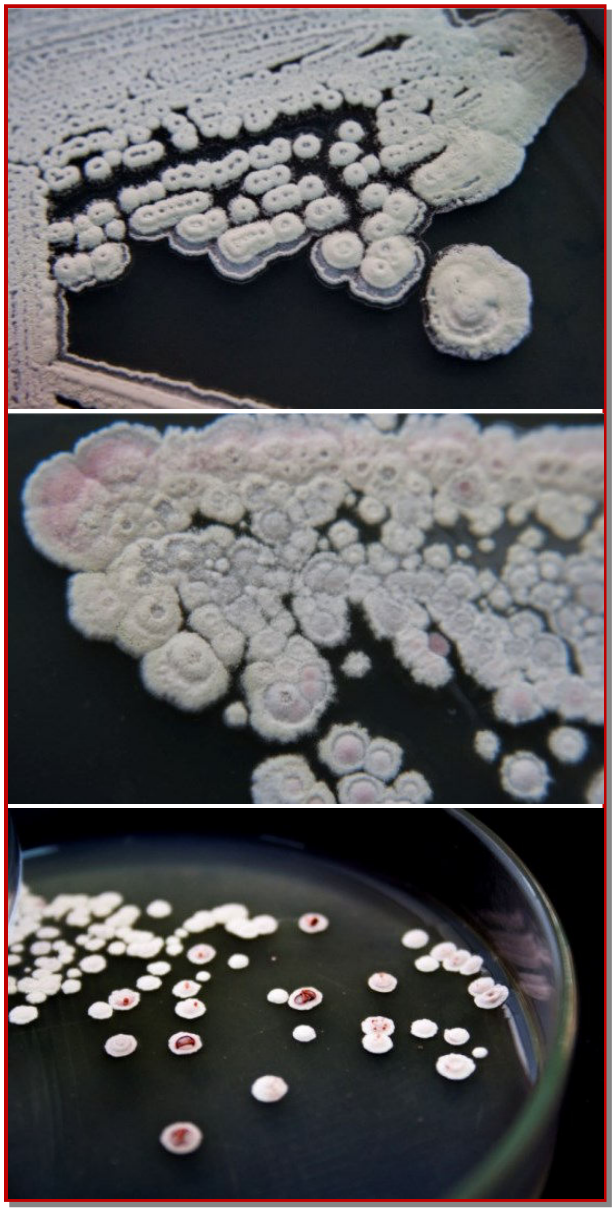

Figure 1: Pure cultures of the marine isolates (A) *Prauserella marina* JUACT 04, (B) *Streptomyces spiroverticillatus* JUACT 05 and (C) *Streptomyces sindenensis* JUACT 06

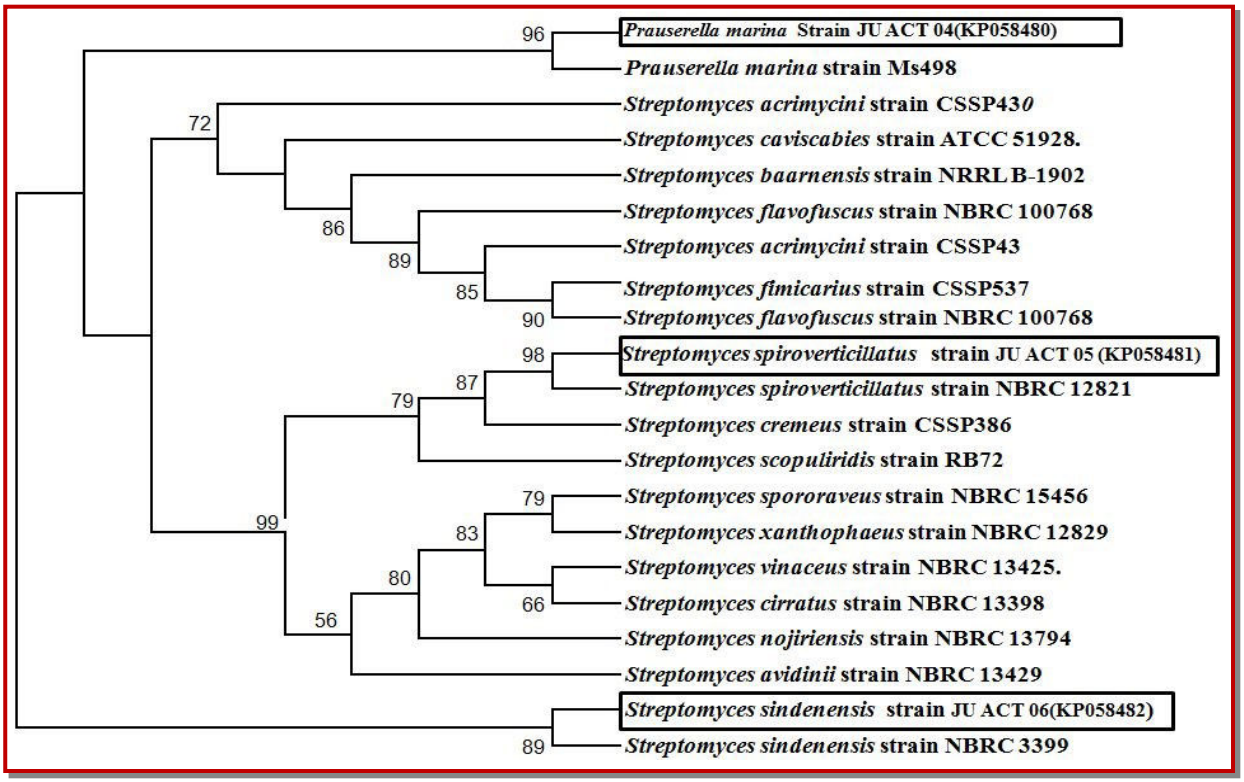

Figure 2: Molecular phylogenetic analysis by maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The analysis involved 21 nucleotide sequences. Evolutionary analyses were conducted in MEGA6

Table I

Antimicrobial activity of the extracts

Test pathogen	Zone of inhibition (mm)		
	Extract from *P. marina* JUACT 04	Extract from *S. spiroverticillatus* JUACT 05	Extract from *S. sindenensis* JUACT 06
Staphylococcus aureus	22 ± 0.2	10 ± 0.3	10 ± 0.2
Staphylococcus citreus	16 ± 0.1	10 ± 0.2	10 ± 0.1
Bacillus cereus	28 ± 0.1	10 ± 0.2	26 ± 0.2
Escherichia coli	24 ± 0.4	Nil	22 ± 0.3
Proteus mirabilis	20 ± 0.2	Nil	18 ± 0.2
Salmonella	15 ± 0.2	10 ± 0.1	10 ± 0.1
Klebsiella	10 ± 0.1	Nil	18 ± 0.1
Bacillus polymyxa	20 ± 0.1	11 ± 0.4	12 ± 0.2
Pseudomonas aeruginosa	20 ± 0.1	11 ± 0.2	26 ± 0.2

the growth of one or the other pathogenic bacteria tested. This was indicated by the clearing zone near the Actinomycetes steaks. The ethyl acetate extracts when tested against bacterial human pathogens resulted in zones of inhibition ranging from 10 mm diameter to 28 mm diameter, indicating potential antibacterial properties of the extracts. It was found that the extract from *P. marina* JUACT 04 and *S. sindenensis* JUACT 06 inhibited the growth of all the nine human pathogenic bacteria tested. The extract from *S. spiroverticillatus* JUACT 05 showed poor inhibitory effect on the growth of pathogenic bacteria indicated by small zones of inhibition. Also, the extract did not show any inhibitory effect on the growth of *E. coli, Proteus mirabilis* and *Klebsiella* clearly indicated by no zone of inhibition (Table I).

In case of cytotoxicity screening using MTT assay, the extract from *P. marina* JUACT 04 alone was able to inhibit the growth and viability of the human cervical cancer cell line HeLa. Further, the TLC fractionation of the extract resulted in three fractions among which the fraction with Rf 0.8 value showed notable cytotoxicity.

The percentage viability of HeLa cells was found to be 23, 32, 46 and 60% with 15, 10, 5 and 2.5 µg/mL

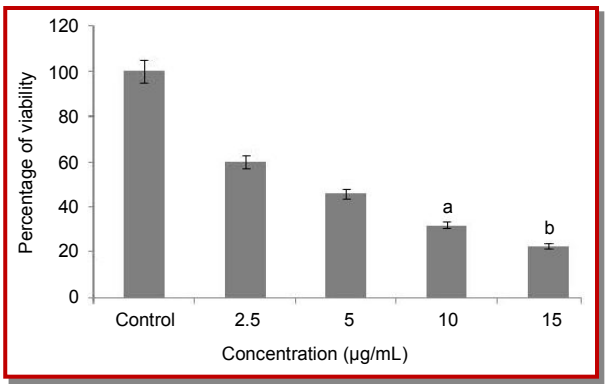

Figure 3: Effect of TLC purified fraction with Rf value 0.8 from *P. marina* JUACT 04 on HeLa cells analyzed by MTT Cell viability assay; [a]p<0.05, [b]p<0.005

for novel therapeutic agents to fulfill the unmet medical needs (Wright and Sutherland, 2007). Marine environments form an excellent source for vast variety of microorganisms. Actinomycetes are one such group in marine ecosystems that are up-and-coming as a source of novel bioactive compounds. Actinomycetes are widely distributed in different marine ecosystems like deep sea floor, surface of the oceans such as in the near shore and intertidal environments, marine sediments, coral reefs, invertebrates and marine plants (Jensen et al., 2005). Marine Actinomyceteshave evolved historically resulting in great metabolic and genomic diversity. The exploitation of marine Actinomycetesas a source for novel antibiotic and cytotoxic compounds is still in its infancy. *P. marina* JUACT 04 isolated from marine sediments is promising as it is found to possess

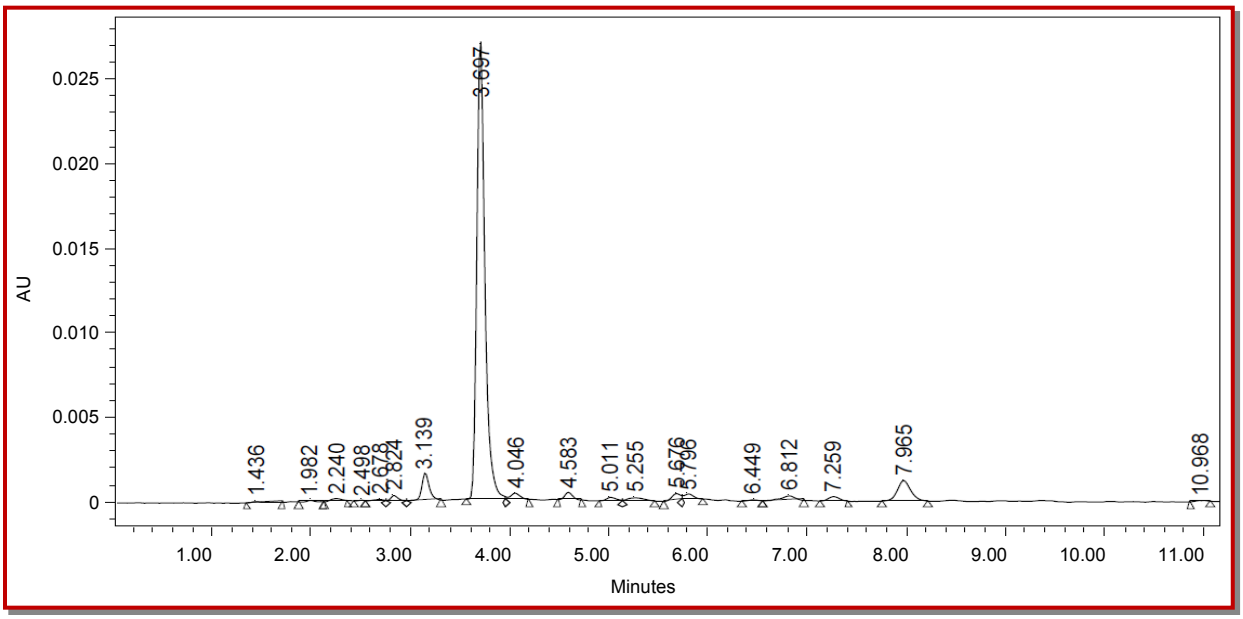

Figure 4: HPLC analysis of bioactive fraction from *P. marina* JUACT 04

(Figure 3). The IC_{50} value is found to be 3.3 µg/mL indicating anticancer potential of the fraction. In an attempt to further purify and characterize the active component from the TLC fraction, HPLC was performed for the third fraction of the ethyl acetate extract from *P. marina* JUACT 04. The HPLC run resulted in a single large peak with a retention time of 3.7 min. The results of the HPLC run indicate that the TLC fraction was consider-ably pure with the major peak contributing to 96.8% of the area (Figure 4).

Discussion

Among the 30,000 diseases which are described clinically, less than one third of them can be treated symptomatically and very few can be cured (Subramani and Aalbersberg, 2012). Currently there is an urgent need antibiotic and cytotoxic potentials. A similar organism *P. marina* sp MS498[T] is also found to exist in the ocean sediment of South China Sea (Wang et al., 2010). It is known that rare Actinomycetes produce diverse and unique, extraordinary sometimes very complicated compounds showing excellent bioactivity and usually low to toxicity (Berdy, 2005). Frigocyclinone, glaciapyrroles, gutingimycin and himalomycins are few of the antibacterial compounds that have been discovered from marine *Streptomyces* sp (Bruntner et al., 2005).The antimicrobial activity of *S. spiroverticillatus* has been reported and is popularly known to produce tautomycin. Tautomycin is a protein phosphatase inhibitor and is structurally close to tautomycetin, a lead for cancer drug. Similarly, the antimicrobial potentials of *S. sindenensis* have been evaluated and found to have significant antibiosis against certain common human bacterial and fungal pathogens (Ningthoujam et al.,

2009). With these findings we conclude that *P. marina* JUACT 04 and *S. spiroverticillatus* JUACT 05 isolated from marine sediments have proven to possess bioactivity. These results provide a justification for further research to purify and evaluate the structural features of the bioactive principle.

Acknowledgement

The authors wish to sincerely thank the management of Jain Group of Institutions for the infrastructural facilities provided to carry out the work.

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