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Antimicrobial and Cytotoxic Potential of the Compound Secreted by Marine Bacteria Collected from the Karwar Coast, West Coast of India.

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Abstract

Antibacterial substances from sea sediment were isolated from marine environment of Karrwar, west coast of India and characterized. Out of the 21 isolates subjected to secondary screening, 10 isolates were active against *Bacillus subtilis,* 12 against *Staphylococcus aureus*, 6 against *Escherichia coli*, 3 against *Proteus vulgaris* and 4 against *Salmonella typhi*. Metabolites in the broth extract of overnight grown *Pseudomonas spp*. No.7 proved to have antimicrobial and cytotoxic against human lung carcinoma cell.

Keywords : Marine Bacteria, antimicrobial activity, cytotoxic activity, human lung carcinoma cells.

Introduction

The study of marine bacteria and their potential role in the production of metabolites is becoming a new topic for research (Faulkner,2000). Several investigations have supplied an increasing number of biologically active and structurally unique compounds (Barsby *et al*., 2001; Hardt *et al*., 2000). Bacteria and other micro-organisms are ubiquitous in the marine environment. They are taxonomically diverse, biologically active, and colonize all marine habitats, from the deep oceans to the shallowest estuaries. It has been estimated that the majority of (Rheinheimer, 1992) bacteria in natural aquatic ecosystems are organized in biofilms. In a biofilm, a microbial community is (Donlan *et al*., 2002) attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances. This structure provides the bacteria present in the biofilm with several advantages compared to those living as planktonic cells. First, the bacteria are maintained in the selected micro environment where population survival does not depend on rapid multiplication. This is especially advantageous in (Jefferson, 2004)environments where the bacteria are exposed to constant liquid movements. Additionally, the bacterial cells present in a biofilm have an increased resistance to desiccation, grazing, and antimicrobial agents compared to their planktonic counterparts. Also, biofilms offer (Fux *et al*., 2005) enhanced opportunities for interactions such as horizontal gene transfer and co-metabolism (Sørensen *et al*., 2005). The occurrence of large scale of bioactive compounds is not common to all living organisms, but restricted to certain taxonomic groups. Recent research progresses reported that many bioactive natural product s from marine invertebrates have striking similarities to metabolites of their associated microorganisms including bacteria. Compared with (Radjasa *et al*., 2007) terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger (Schwartsmann *et al*., 2001). Competition among microbes for space and nutrient in marine environment is a powerful

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selection pressure that endows marine micro-organisms to produce marine natural products possessing medical and industrial values. Many antimicrobial substances (Armstrong *et al.*, 2001) have been found among these kinds of bacteria due to the specialized role they play in their respective hosts. It is suggested that the primary role of these (Holmstrom *et al.*, 2002) antibiotic substances could be related to ecological competition (Burgess *et al.*, 2006). The aim of this study was to investigate the antimicrobial and cytotoxic activity of Pseudomonas species isolates from marine environment of Karwar coast, West coast of India.

Materials and Methods

Sample collection

The sea sediment and water sample were collected from the Karwar region, west coast of India (Lat. 14° 47' 11.33N. Long. 74° 01' 48.38E). The samples were brought to the laboratory in aseptic condition. Than the microorganisms were cultivated on Zobell Marine Agar 2216, than it was sub cultured on Modified Nutrient agar (MNA).

Screening of Isolates for antimicrobial activity

In preliminary screening, determination of the antimicrobial activity of pure isolates was done by zone of inhibition method on Nutrient agar (NA) using *Salmonella typhi* as pathogen. Further screening was performed by zone of inhibition method against the standard test organisms. Active microbes were cultured for the screening of antibiotic substances in nutrient agar at 28°C for overnight. The test organisms used were: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klebsiella spp., S. typh*i and *Enterococcus faecalis*. (Figure 1)

Characterization of isolates

The potent isolates selected from the secondary screening were characterized by morphological and biochemical methods. The results of microscopic examination were compared with Bergey's manual of Determinative Bacteriology, Ninth edition (2000) and the organism was identified. Various biochemical tests were performed for the identification of the potent isolates are as follows; Fermentation of sugars, Hydrolysis of starch, Indole production, Methyl red, Vogues Prauskauer, Citrate utilization, Urease test, 2%Peptone water, Nitrate reduction test, Gelatin liquefaction, Catalase test, Oxidase test.

Isolation and purification of antibacterial metabolites

Samples from *Pseudomonas* Culture No.7 grown in 1 L nutrient broth at 28°C and 220 rpm were taken and extraction was carried out using ethyl acetate solvent extraction [ethyl acetate : filtrate 1:1 (v/v)] on shaker at 220 rpm for 1 hr. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase and by evaporation in water bath at 80-90°C the residue was obtained and weighed. The compound thus obtained was used to determine antimicrobial activity and minimum inhibitory concentration. The antimicrobial activity was determined by spectrophotometric method. The residue obtained was dissolved in 1 ml 0.2 M phosphate buffer (pH 7.0). 100 µl of preparation was loaded into a well in the 24 well plates, containing pathogenic cultures and were incubated at 37°C for 18 - 48 hr and optical densities measured at 540 nm using ELISA plate reader (Figure 1). For thin layer chromatography (Becker *et al.*, 1964) silica gel plates (Merck) 5 X 20 cm, 1 mm thick were used. 10 µl of the ethyl acetate fractions and reference antibiotics were spotted on the plates and the chromatogram was developed using ethylacetate: iso-propanol: acetonitrile (1:4:5) as solvent system.

Cytotoxicity assay

Human Lung carcinoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). For cytotoxicity assays, cells were seeded into 24-well plates at a density of 2.1 × 105 cells/well and the dilutions (1:500 and

1:1000) of the extract made in DMEM in the volumes of 10 µl and 25 µl were added. After incubation period (24, 48 h), the growth media was removed and the cells were trypsinized and counted with a Hemocytometer (Freshney *et al.*, 2006).

Results

Out of 38 isolates subjected for primary screening process, only 21 isolates showed activity against test organisms. Of which, only 3 were active against gram negative organism, 09 against gram positive organisms and 09 against both gram positive and gram negative organisms.

Out of the 21 isolates those were subjected for the secondary screening, 10 isolates were active against *B. subtilis,* 12 against *S. aureus*, 6 against *E. coli*, 3 against *P. vulgaris* and 4 against *S. typhi*.It can be seen that the Pseudomonas culture no.7 shows maximum zone of inhibition against pathogenic culture. Culture no 7 which showed activity against all pathogenic strains (Figure 1).

Figure 2 shows the effect of extract of 48 h grown culture of *Pseudomonas* spp. colony 7 on various pathogenic cultures in the form of optical densities obtained. Identification of the potent antibiotic producing strains reveals that most of the specimens belong

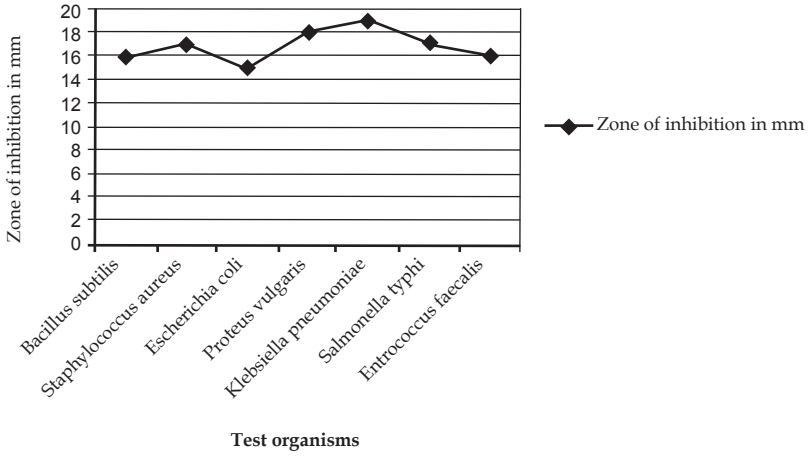

Figure 1**.** 25 µl of overnight grown culture of *Pseudomonas* spp.no.7 shows antimicrobial activity against all pathogenic strains.

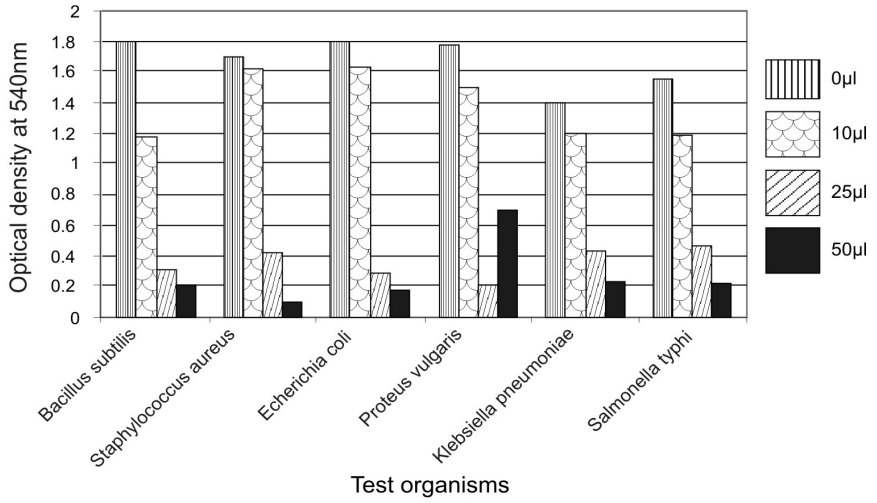

Figure 2. Effect of extract of overnight grown culture of *Pseudomonas* spp. No. 7 on various pathogenic cultures.

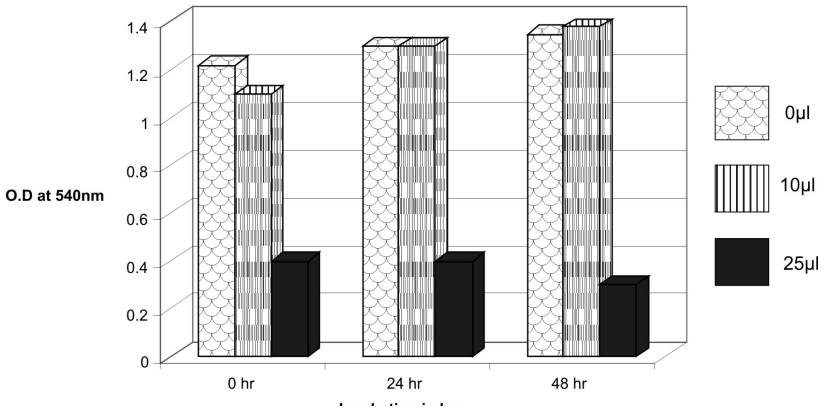

Figure 3. Cytotoxic effect of extract of 48 h grown culture of *Pseudomonas* spp. no.7 on Lung carcinoma cells.

to the genus *Pseudomonas*. The minimum inhibitory concentration of the extract from *Pseudomonas* spp. no.7 was 0.73 mg.ml^{-1}. Also the results from thin layer chromatography showed the spot given by the extract of *Pseudomonas* spp. was with Rf value 0.78.

We examined the effect of *Pseudomonas* spp. and its metabolites on the growth of eukaryotic cells. For the cytotoxicity studies human lung carcinoma cell were chosen. Monolayer cultures of human lung carcinoma cells were exposed to various concentrations of the extracts and cell viability evaluated by counting live cells after 24 and 48 h of exposure (Figures 3). The extract caused cell death at less dilutions of 1/500 (10 µl). At dilution of 1/1000 (25 µl) cell viability greatly reduced after 48 h of exposure.

Discussion

The putative isolates of primary screening when subjected to secondary screening, showed different activity from that of primary screening; some of the active isolates didn't show the activity in the secondary screening while some showed little activity and some showed improved activity. According to Bushell (1993), during the screening of the novel secondary metabolite, Marine isolates are often encountered which show antibiotic activity on agar but not in liquid culture.

The result of primary and secondary screening revels that most of the active isolates were active against gram-positive bacteria (*Bacillus subtilis* and *S.aureus*) than gram-negative bacteria. The reason for different sensitivity between gram positive and gram-negative bacteria could be attributed from the morphological differences between these microorganisms, gram-negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes; the gram positive should more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer *et al.*, 1971).

Although various biochemical tests were performed, further 16S rRNA sequencing has to be taken for identification at species level. The minimum inhibitory concentration (MIC) for the antimicrobial extracted from *Pseudomonas* spp. no.7 was 0.73 mg.ml^{-1}. This shows that the antimicrobial from *Pseudomonas* spp. no.7 was more active against the pathogenic organisms but there are various factors affecting the activity.

The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration. For complete characterization of an antibiotic it should be isolated in pure form as a single component but this is impractical in a screening programme like this. However, a little effort was made in this approach.

According to the TLC separation, the two extracts yielded components with Rf values similar to the antibacterial compounds as visible on bioautogram. In addition, the inhibition zones were associated with yellowish green spots, which had been detected under UV radiation. This may mean that the same compounds are responsible for antibacterial activity of those isolates.

The effect of *Pseudomonas* spp. and its metabolites on the growth of eukaryotic cells has been noted . For the cytotoxicity studies human lung carcinoma cell were chosen. Monolayer cultures of human lung carcinoma cells were exposed to various concentrations of the extracts and cell viability evaluated by counting live cells after 24 and 48 h of exposure (Figures 3). The extract caused cell death at less dilutions of 1/500 (10 µl). At dilution of 1/1000 (25 µl) cell viability greatly reduced after 48 h of exposure.

Although the antimicrobial and cytotoxic agents obtained in this study can not be declared as new bioactive compound, there is the probability of finding new bioactive compound in Karwar because of its wide biodiversity. For proper identification of the extracts of bioactive compound, it is necessary to obtain in pure form, which requires a series of purification process and different chemical analysis. As we know the land of Karwar is virgin in this field, so lots of works should be done to explore the new bioactive compound because any new bioactive compound and its producing organism have been a great demand.

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