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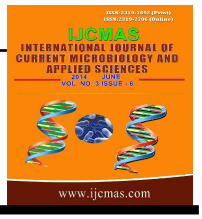

Original Research Article

Isolation of Secondary Metabolite From Seawater Bacterial Population and Screening of their Bioactive Potential Against Urinary Tract Pathogens Sourced From HIV Infected Patients

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A B S T R A C T

The marine environment may contain over 80% of world's plant and animal species. Marine water have been reponed to possess a wide range of bio active properties. In recent years, many bioactive compounds have been extracted from various marine plants like seagrass ,seaweeds and marine organisms. The search for new metabolites from marine organisms has resulted in the isolation of more or less10,000 metabolites, many of which are endowed with pharmacodynamic properties. From this present study the bacteria were isolated from deep seawater along the East Coast of India, Palk Strait to find out the potential of antibacterial activity. Based on the morphological characters, 36 different strains were isolated. Based on the effect of bioactive compound chosen bacterial strains were assayed for antibacterial effect through agar well diffusion method and tested for the antimicrobial sensitivity against *Staphylococcus aureus, Escherichia Coli, Klebsiella pneumonia and Pseudomonas aeruginosa Sp* . Of them, 12 strains were showed sensitivity against two pathogenic bacteria Escherichia Coli, Klebsiella pneumonia which were subjected for the antimicrobial potential of the marine microbes . The Fraction I, Fraction II, Fraction III shows the greater effect against the clinical pathogens *Escherichia coli and Klebsiella pneumoniae*. Fraction III shows greater zone of inhibition. From the curve of elution in Anion exchange chromatography (DEAE Sepharose Fast Flow chromatography). Three A280 nm peak

Keywords

Sea water, Bacterial Strains, Antimicrobial Activity, Agar Well diffusion assay, Anion exchange chromatography

Introduction

The world's oceans, covering more than 70% of the earth's surface, represent an enormous resource for the discovery of potential chemotherapeutic agents. Taking higher Taxonomic levels as an estimate of biodiversity, more phyla are found in the oceans than on land. Of the thirty three known phyla of animals, only one is exclusive of land, while as many as twenty-one phyla are exclusive of the sea (Pietrae F, 2002).

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phyla of animals, only one is exclusive of land, while as many as twenty-one phyla are exclusive of the sea (Pietrae F, 2002). 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 environments where the bacteria are exposed to constant liquid movements, as, for example, in aquatic environments. 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 enhanced opportunities for interactions such as horizontal gene transfer and co-metabolism.

The nutritional and environmental conditions have a great influence on production of the antimicrobial substances. In order to develop an efficient production of antimicrobial substances, knowledge regarding the environmental factors affecting this process needs to be well identified. Experimental designs are excellent techniques for optimization of culture conditions to achieve optimal production. Marine microorganisms which are salt-tolerant provide an interesting alternative for therapeutic purposes.

Marine microorganisms have a diverse range of enzymatic activity and are capable of catalysing various biochemical reactions with novel enzymes. Especially, halophilic microorganisms possess many hydrolytic enzymes and are capable of functioning under conditions that lead to precipitation of denaturation of most proteins. Further, it is believed that sea water, which is saline in nature and chemically closer to the human blood plasma, could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans(Piel J. et al., 2004).

The increasing prevalence of multidrug-resistant bacteria has made the search for new antimicrobial agents an important strategy in the establishment of alternative therapies for difficult-to-manage infections (Rice 2006). Microorganisms produce many of the natural products isolated from marine invertebrate sources. The extensive association between marine invertebrate species and marine micro biota is remarkable. For instance, bacteria can constitute up to 40% of the total weight of some sponges. Sponges and other marine invertebrates harbour diverse microorganisms and benefit from the rich source of chemicals produced by microbes, which can protect these sponges or other hosts against predators or pathogens (Gerwick et al., 2008). The large number of strains recovered with antimicrobial activity suggests that marine represent an ecological niche harboring a largely uncharacterized microbial diversity and as yet unexploited potential in the search for new secondary metabolites.

This offer the possibility of using sponge-associated bacteria instead of the sponge itself for the production of biologically active substances. Since bacteria rapidly produce large quantities of biomass, biologically active products can be easily obtained on a biotechnological scale without the need for cultivating or harvesting the sponges. In many instances, the limited availability of sponge material may preclude the commercial production of bioactive compounds.

As the study of marine bacteria and their potential role in the production of pharmacologically active metabolites is a

promising new topic for research, in the present study we isolated and characterized bacteria with antimicrobial activities from clinical pathogens.

Urinary tract infection In- Men with AIDS

The Acquired immunodeficiency syndrome (AIDS) has predominantly occurred in young adult or middle aged males, a population otherwise expected to be at very low risk for urinary tract infection (UTI). However, some uncontrolled studies have suggested that men with AIDS may be at increased risk for bacteriuria. Recently, a controlled longitudinal study showed that men infected with the Human immunodeficiency virus (HIV), the causative agent of AIDS, who had CD4+ lymphocyte counts below 200 cells/mm3 were at increased risk for bacteriuria when compared with other HIV-infected men but with higher CD4+ lymphocyte counts.

In spite of this elevation in the risk for bacteriuria, the frequency of symptomatic UTI has not yet been shown to be significantly increased among HIV-infected men, in comparison with a similar population without HIV infection. Furthermore, Welch et al suggested that most cases of asymptomatic bacteriuria in HIV infected men may be self-limited. Thus, clinical relevance of UTI as a cause of morbidity in male patients with HIV infection has not been established.

To investigate the frequency of bacteriuria and of symptomatic UTI in adult males with HIV infection, we conducted a controlled, cross-sectional study, comparing a group of men with AIDS with a group of male patients without HIV infection, both studied within 24 hours after hospital admission. A group of male outpatients with asymptomatic HIV infection was also studied. (Ana Maria Felix De Pinho., 1994)

Antibiotic resistance in urinary tract infections

The majority of isolates was resistant to amoxicillin and co-trimoxale, and the proportion of resistant *Escherichia coli* isolates increased during the study period. In a prospective 4-month study in 1991 we found that the vast majority of isolates was susceptible to aminoglycosides, amoxicillin/ clavulanate.

Materials and Methods

Description of the Study area

Thondi is situated in the Palk Strait region of Tamil Nadu. The study area lies in the latitude of 99°44”N and 79 10’ 45” E. the marine water were collected from Deep Sea during month of (December 2014)

Isolation of marine organism

Isolation using differential media the water sample was serially diluted and spreaded into the Zobell marine agar medium. After incubation period the bacterial species enumerated on the Zobell marine 2216e (Hi-media) agar medium were counted and the total number of bacterial counts was expressed as Colony Forming Unit (CFU).

The colonies were counted and the dominated colonies were picked out and their morphology morphological characterization was studied. The dominated colony were streaked and the pure culture were stored for further studies.

Isolation of HIV infected patients urine associated pathogens

The clean catch of early morning mid stream urine from HIV patients from Nammakal District were collected in a sterile container. The urine samples associated pathogens were isolated by serial dilution method. The chosen isolates were identified using standard staining and biochemical test. (Bergey's manual Holt et al., 1994).

Extraction of total proteinsMarine bacteria isolated were cultured in 300 ml Marine FePO4 0.1 g, dissolved in (seawater, pH 7.2–7.6) for the production of bioactive compounds in 500 ml Erlenmeyer flasks. Flasks were incubated on a rotatory shaker at 220 rev/min at 25 ℃. After 7 days of cultivation. The culture was centrifuged. After centrifugation (16,000 g, 20 min) at 4 °C, the supernatant was collected and the crude extract obtained. The crude extract of bacteria was fractionated by salting out with increasing concentrations of ammonium sulfate. Solid ammonium sulfate was slowly added to the above crude extract with gentle stirring, up to 35% saturation in 20 min. After the crude extract was left at 4°C with the ammonium sulfate under vortexing for another 40 min, the protein precipitate was collected by centrifugation (16,000 g, 20 min) at 4°C. The supernatant was transferred to another beaker, and solid ammonium sulfate was added to it up to 70% saturation.

The mixture was treated as above. Likewise, another protein precipitate was obtained at 70%−100% saturation of ammonium sulfate. Each of the three protein pellets was suspended in 10 ml of ice-cold PBS (10 mM, pH 8.0), and dialyzed against a large volume (3 L) of distilled water for 24 h at 4 °C. Dialysis bags were employed. During this process, the dialysate was changed three times to completely remove any residual ammonium sulfate. Antimicrobial Activity by Agar Well Diffusion Assay (Perez et al., 1990). The agar well diffusion method was used for the inhibitory effects of crude extract of marine organism against the clinical pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia* extract was loaded on the Muller Hinton agar plates.

Five wells (6 mm in diameter) were made equidistance in each of the plates using a sterile cork borer. Up to 25µl to 100 µl of each concentration of the extract were respectively introduced into the wells using sterile automatic pipettes, with the stock in one well. It was allowed to diffuse at room temperature for 2 hrs and the plates were incubated to 37°C for 24 hrs. Diameters of the inhibition zones were measured. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by marine microbe..

Purification of proteins

Anion exchange chromatography (DEAE Sepharose Fast Flow): The Fraction-I, Fraction-II, and Fraction-III were obtained at the ammonium sulfate saturation of 0-35%, 35-70% and70-100%, Fraction III were active in the antimicrobial activity. Fraction III were obtained from the crude extract at 70%−100% saturation of ammonium sulfate was dialyzed against 10 mM Tris-HCl, pH 7.46 for 5 h and the dialyzed solution was subsequently injected into a DEAE Sepharose Fast Flow column, which was pre-equilibrated with the aforementioned Tris-HCl buffer. The column was washed with the same buffer

until the baseline returned to zero and remained stable. The column was then eluted with increasing concentration of NaCl prepared in 10 mM Tris-HCl buffer, pH 7.46 at 4 °C. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Seven A280 nm peak fractions, named G-1, G-2, and G-3 were collected respectively.

Protein assay

The protein content of total protein extract fraction-III, G-1, G-2 and G-3 was determined by lowry's method Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Sodium dodecyl sulfate-polyacrylamide gel electrophoriesis (SDS-PAGE) analysis was Performed (Fraction III and G1).

Results and Discussion

Marine bacteria have been recognized as important and untapped resource for novel bioactive compounds (Anand TP,et al 2006). Development of marine biotechnology is expected to produce novel compounds that may contribute significantly towards drug development over the next decade. In this study, we isolated several marine bacteria (see Methods) for extraction of some possible types of antimicrobial compounds active against drug resistant organisms. To demonstrate and characterize this possible antagonistic ability, antibiotic resistant clinical isolates and indigenous marine bacteria were used as revelator.

In present study the about 36 bacterial strains were isolated from the marine water and all of them have been tested for the antimicrobial sensitivity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa* sp by Agar well diffusion assay. Of them, twelwe strains were shown sensitivity against two pathogenic bacteria *Escherichia coli and Klebsiella pneumonia*. Of the antimicrobial potential of marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect against the clinical pathogens *Escherichia coli* and *Klebsiella pneumonia*.

Fraction III shows greater zone of inhibition. The results suggested that the fraction-III needed further purification in order to unveil the active components of *Escherichia coli* and *Klebsiella pneumonia*. From the curve of elution in DEAE Sepharose Fast Flow chromatography Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. In the previous studies of the Liyan Song et.al 2008 isolated seven fraction from the protein of invertebrates. The antimicrobial potential of the marine microbes the G-1, G-2, G-3 shows the greater effect against the clinical pathogens *Escherichia coli and Klebsiella pneumonia*. Among that G-3 shows the maximum results of 18.2 mm and 15.4 mm against *Escherichia coli* and *Klebsiella pneumonia*. Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3. In the previous (Yamamoto et al., 1994 study the aminoacids were studied and results were similar to the present studies).

In the present studies the crude extract were partially purified by the ion exchange chromatography and the antimicrobial activity was analysed and the compound was considered as the bioactive compound because of the biopotentiaal of the compound against the clinical pathogen (Kondratieva LM, Vakhusheva EV 1991).

Antimicrobial Activity by Agar Well Diffusion Assay (Perez et al., 1990)

The antimicrobial potential of the marine microbes the Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens, *Escherichia coli* and *Klebsiella pneumonia*. Fraction III shows greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl, 100 µl). The higher concentration of 100 µl of sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Escherichia coli* (14mm), *Klebsiella pneumonia* (13mm), which shown in the table. The results suggested that the fraction-III needed further purification in order to unveil the active components of *Escherichia coli and Klebsiella pneumonia.*

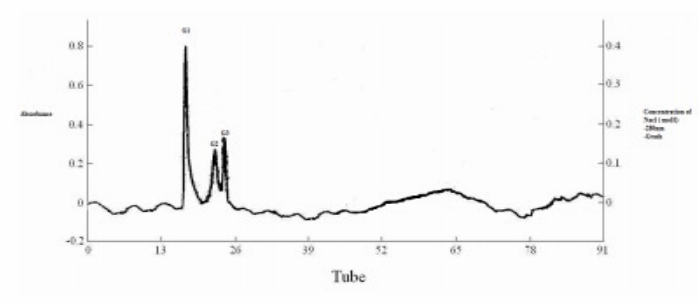
The curve of elution in DEAE Sepharose Fast Flow chromatography

Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10 mM);

Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 mL/min; Collection rate: 5 mL/tube.

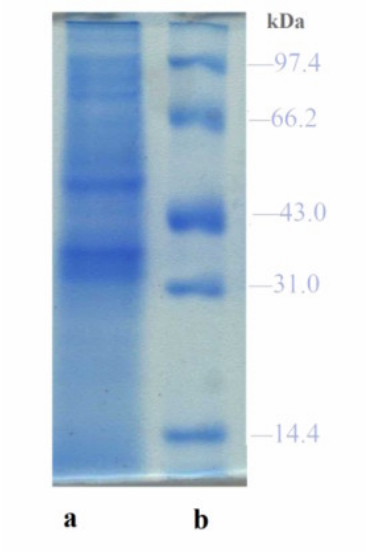
Crude extraction

Name of the organisms	Crude extract exhibit zone of inhibition in mm				
	25 µl	50 µl	75 µl	100 µl	Antibiotic streptomycin 100 µl
Fraction I					
Escherichia Coli	3	3	3.3	4	6
Klebsiella pneumonia	4.2	4.5	4.8	4.8	8
Fraction II					
Escherichia Coli	5	5	6	6.2	6
Klebsiella pneumonia	6	6.3	7	7.2	8
Fraction III					
Escherichia Coli	10	12	12.5	14	6
Klebsiella pneumonia	12	12.5	13	13	8



Characterization of purified proteins

To estimate the molecular weight of G-3, Molecular weight marker (range from 14.4kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46kDa, band 35kDaand band 32kDa.



Life originated in the sea and has sustained itself to the present day. The Marine ecosystem is a rich source biological diversity. This has been exploding in the discovery of unique chemicals having potential for industrial development as pharmaceuticals. In recent years, a significant number of novel secondary metabolites with potential pharmacological properties have been discovered from marine organisms. The world's oceans comprise the largest part of the biosphere and contain the most ancient and diverse forms of life.

The bacterial organisms isolated from seawater. Several studies have indicated that the HIV patients urine sample contain the pathogenic microbes. Reduction of initial bacterial load in urine is of prime importance in an attempt to improve the health of the patient. Hence the present study was undertaken to produce valuable secondary metabolites from the natural marine water against the HIV infected urine pathogens. Based on the morphological characters, 36 strains were isolated and all of them have been tested for the antimicrobial sensitivity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa* sp by Agar well diffusion assay. Generally, the bacteria organism isolated from seawater showed maximum sensitivity against several human bacterial pathogens. The earliest marine bio active compounds were isolated from natural product of marine microorganisms. The bioactive secondary metabolites isolated from seawater are produced by functional enzyme clusters, originated from the seaweeds, seagrass and sponges associated microorganisms. A wide range of chemical and functional diversity has been observed among bioactive compounds. Of the various classes of compounds, polyketides, alkaloids, fatty acids, peptides and terpenes are the most abundant ones.

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