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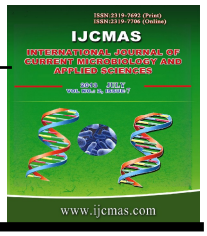

Original Research Article

Antibacterial activity of protein extract of marine *Pseudomonas aeruginosa* against bacterial pathogens

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Antimicrobial substances; *Pseudomonas aeruginosa;* antibacterial activity; antimicrobial resistance.

A B S T R A C T

Antimicrobial substances are so widespread that they are likely to play an important protective role. Marine bacterium has been recognized as important antimicrobial substances producers which have an exceedingly bright future in the discovery of life saving drugs. Wet soil samples were collected from marine resource for the isolation of bacteria. 4 bacteria's of *Pseudomonas* were isolated based on their morphological and structural characteristics. They were screened for antibacterial activity against bacterial pathogens by point inoculation and agar well diffusion method. Screening for antibacterial activity of the supernatant of A was also done. In all the screening processes isolate showed zone of clearance of 12mm. The marine isolate was identified by as *Pseudomonas aeruginosa.* From the present study it has been concluded that the antimicrobial substances from *Pseudomonas aeruginosa* has greater antibacterial activity against bacterial pathogens than Bacitracin and tetracycline. The findings of this surveillance study will enhance our knowledge regarding the problem of antimicrobial resistance and will serve as a basis for future development of new anti – bacterial pathogens substance against new resistance in bacterial pathogens.

Introduction

Antibiotics are components or substance that kills or inhibits the growth of microbes produced naturally by microbes or chemically synthesized. In the last few years many cationic peptides have been isolated from a wide range of animal, plant, and bacterial species (Aley *et al.*, 1994). These compounds comprise a diverse class of molecules used in host defense by plant, insect, fishes, crustaceans, amphibians, birds, mammals, and humans.

Production of antimicrobial substances seems to be a general phenomenon for most bacteria. A microbial defense system is produced, including broad spectrum classical antibiotics, metabolic by-products such as organic acids, and lytic agents such as lysozyme. In addition, several types of protein exotoxins and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action were described (Riley and Wertz 2002; Yeaman and Yount 2003). They can

be divided into three major classes of which class I and II are quite heat-stable. Class I contains modified bacteriocins, so-called lantibiotics, that are found among many different Gram-positive bacteria but have yet to be found in Gram-negative bacteria. They are divided into two subclasses (a) the linear and cationic peptide and (b) the globular peptides; the latter normally are hydrophobic but not cationic. The second major class (II) contains small heat-stable bacteriocins that lack post translational modifications as found in lantibiotics, and are presently clustered into atleast two groups: pediocin-like bacteriocins and two-peptide bacteriocins (Diep *et al.,* 2002). A third class (III) of bacteriocins has been also defined. They are normally larger in size and are easily subjected to heat inactivation (Klaenhammer *et al.,* 1993).

Hundreds of peptide antibiotics have been described in the past half-century (Honcock *et al.,* 1995). These falls into two classes, non ribosomally synthesized peptides, such as the gramicidins, polymyxins, bacitracins, glycopeptides, etc., and ribosomally synthesized peptides. Nonribosomally synthesized peptides can be described as peptides elaborated in bacteria, fungi and actinomycete that contain two or more moieties derived from amino acids (Kleinkauf *et al.,* 1988). Antimicrobial, ribosomally synthesized, cationic peptides have been recognized only recently as an important part of innate immunity (Bevins, 1994).

The antimicrobial peptides are found in animal tissues exposed to microbes or cell types that are involved in host defense. Epithelial surfaces secrete antimicrobial peptides from both barrier epithelia and glandular structure (Zasloff, 1987; Diamond *et al.,* 1991; Ouellette and Selsted, 1996). Phagocytic cells contain several types of storage organelles for microbicidal substances and digestive enzymes.

As current antibiotic therapy options are becoming limited for staphylococcal infections, there is an urgent need for new antimicrobial agents to combact these resistant pathogens. Antimicrobial peptides are now promising class of antimicrobial agents derived from naturally occurring peptides (Hanoc, 1997). The marine surface environment is a site of intense competition for living space by a wide variety of organisms. Bacteria are generally recognized as primary colonizers of this habitat (Bryers, 1982). The microbial diversity in the sea is yet to be revealed. In the last few years marine microorganisms emerged as a new field for the discovery of novel biologically active compounds (Fenical, 1997). Marine natural products have an exceedingly bright future in the discovery of life saving drugs. The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder *et al*, 1996).

Materials and Methods

Sample collection

Wet soil samples were collected from Mammallapuam. The five sampling spot were selected for the isolation of bacteria which have antibacterial activity. The samples were collected in freshly purchased polythene bags, sealed properly and were brought to laboratory thereby preventing any contamination on the way. The samples were then stored at 4˚C until used to minimize the metabolic activities of microorganism and to keep them in their exact qualitative and quantitative level of population.

Isolation of bacteria

Serial dilution agar plating method (or viable plate counting method) was followed to isolate bacteria from sample using Zobell marine agar.

Identification of bacteria *P.aeruginosa*

Presumptive *P.aeruginosa* colonies were then subjected to Gram 's staining and a series of biochemical tests such as urease test, indole production, methyl red test, Voges Proskauer test, and cultures which matched typical reaction of standard were confirmed as *P.aeruginosa*.

Collection of pathogenic bacteria

The clinical isolate of *Vibrio* spp. *E.coli, Klebsiella pneumonia, Salmonella typhi, S. aureus, E. faeclais, Proteus vulgaris,* collected from Dept. of Microbiology, Kanchi Shri Krishna College, Kancheepuram, Tamil Nadu and was preserved in agar slants for further use and stored at 4˚ C.

Antibiotic sensitivity test

Then Kirby – Bauer method (agar diffusion method) was followed to determine the susceptibility of clinical isolate of bacterial pathogens to antibiotics.

Screening of marine isolates for its antibacterial activity

Point inoculation method

Muller Hinton Agar plates were prepared. The overnight culture of clinical isolate of bacterial pathogens in nutrient broth was uniformly swabbed on the surface of Muller Hinton Agar plates using sterile cotton swab, making a lawn culture. The marine isolates from the agar slants were spotted on to Muller Hinton Agar plates seeded with actively growing cells of the clinically isolated MRSA. The plates were incubated at 37˚C for 24 hours. After the incubation period, the plates were observed for the zone of inhibition and the results were recorded.

Agar well diffusion method

After screening by point inoculation method, the antimicrobial substance production of the marine isolates was tested by agar- well diffusion method.

Muller Hinton agar plate was prepared. The overnight culture of clinical isolate of bacterial pathogens in nutrient broth was uniformly swabbed on the surface of Muller Hinton agar plates using sterile cotton swab. Two wells of 3mm size were made with sterile cork borer on the seeded plate. Around 50µl of overnight broth culture of marine isolate was added to the well aseptically. The plates were incubated without inverting for 24hrs at 30°C and the zone of inhibition was observed and recorded.

Screening of marine isolate's supernatant for its antibacterial activity

The overnight grown culture of marine isolate in nutrient broth was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. The overnight culture of clinical isolate of bacterial pathogens in nutrient broth was uniformly swabbed on the surface of sterile Muller Hinton agar plate using cotton swab. Two wells of 3mm size were made with sterile cork borer on the seeded plate. Around 50µl of overnight broth culture of marine isolate was added to the well aseptically. The plates were incubated without

inverting for 24hrs at 30°C and the zone of inhibition was observed and recorded.

Protein precipitation by ammonium sulphate method

An overnight broth culture of Sample was centrifuged at 6000 rpm for 10 minutes at 4°C. To the cell free supernatant, ammonium sulphate was added to achieve 30% saturation. It was stirred well and incubated at 4°C overnight. The following day, the precipitates were collected by centrifugation at 6000rpm for 25 minutes at 4°C. The precipitates were dissolved in 500µl of sterile distilled water. Antibacterial activity of both the supernatant and the precipitate were tested using agar well diffusion assay and the results were recorded.

Protein precipitation by TCA method

One volume of TCA was added to four volumes of protein samples.i.e. 1.5.ml tube with maximum volume of 1ml sample, 250 ul of TCA was added. The tube was incubated for 10 minutes at 4°C. The tube was centrifuged at 500 rpm for 5 minutes. Supernatant was removed, leaving protein pellet intact. Pellet should be formed as whitish, fluffy protein. Pellet was washed with 200ul cold acetone. The tube was centrifuged at 5000 rpm for 5 minutes. The steps were repeated for 4-6 times with total of 2 acetone washes. The pellet was dried for 5-10 minutes to drive off acetone. Antibacterial activity of dried pellet was checked and results were noted

Protein precipitation by Ethanol: choloform method

To 100 ul of sample, 400 ul of ethanol, 100 ul of choloform and 300 ul of distilled water were added and vortexed thoroughly and then placed on ice for 2 minutes. Then the tubes were centrifuged for 2 minutes at 10000 rpm. Supernatant was removed. To the pellet 300 ul of ethanol was added and vortexed thoroughly. The tube was centrifuged for 5 minutes at 10000 rpm. Supernatant was removed and the pellet was dried for further use. Antibacterial activity of dried pellet was checked and results were noted.

Results and Discussion

Isolation of Marine bacteria *P. aeruginosa*

Marine bacteria were isolated from five wet soil samples from Mammallapram using Marine agar 2216 by serial dilution spread plate method. A total of 4 bacterial strains of *P. aeruginosa* were isolated based on their morphological and structural characteristics (Table.1).

Antimicrobial susceptibility of clinical isolate of bacterial pathogens to antibiotics

Antimicrobial susceptibility of clinical isolate of bacterial pathogens, Co-metrizole, Amikacin, Bacitracin, Gentamicin, oxytetracycline was carried out by Kirby Bauer disc diffusion assay. The results were tabulated (Table 2) .

Screening of marine isolates for its antibacterial activity

All of the marine isolates were tested for their ability to produce antibacterial substance against clinical isolate of bacterial pathogens. Marine isolates from agar slants were spotted onto the Muller Hinton agar. Out of the 4 isolates, only one isolate *P. aeruginosa* showed inhibitory activity against clinical isolate

of bacterial pathogens. Zone of clearance of about 12mm was observed after 24 hours of incubation.

Table.1 Identification of Marine isolates of *P.aeruginosa*

S.No	Biochemical tests	Results
1	Gram staining	Gram negative
	Pigment production	+
2	Motility	Motile
3	Indole	-
4	Methyl red	-
5	Vogeus Proskauer	-
6	Citrate Utilization test	+
7	Catalase	+
8	Urease	+
9	Nitrate reduction test	+
10	Oxidase test	+
11	Sugar Fermentation test Glucose Maltose Sucrose Mannitol	 - + - -
12	H2S production	-

After screening by point inoculation method, the antibacterial substance production was confirmed by agar well diffusion method. The overnight culture of isolate *P. aeruginosa* was added to the wells in Muller Hinton agar (MHA) plate. A clear zone of inhibition of 35mm was observed against *Vibrio* sp. 1. and 25mm in *S.typhi* (Table 3).

By Culture agar well diffusion method, the antibacterial substance production was confirmed by agar well diffusion method. The overnight culture of isolate A was added to the wells in Muller Hinton agar (MHA) plate. A clear zone of inhibition of 17mm was observed against observed against *Vibrio* sp. 1. and 15mm in *Vibrio* sp. 2. (Table 4).

Screening of marine isolate's supernatant for its antibacterial activity

To check whether the antibacterial substance produced by isolate A is an extracellular compound, the supernatant of *P. aeruginosa* was tested for its antibacterial activity by agar well diffusion method. Zone of clearance of 125 2mm against *Vibrio* sp. 1 and 23 mm in *S.tpyhi* (Plate 5).

Antibacterial activity of the protein precipitated by ammonium sulphate method

The protein precipitated by ammonium sulphate method was dissolved in sterile distilled water and was desalted. Antibacterial activity of the precipitate protein and the supernatant was checked. A clear zone of 16mm was noted around the well loaded with the protein sample against *S.aureus* . No zone of clearance was seen around the well loaded with the supernatant (Table 6).

Antibacterial activity of the protein precipitated by TCA method

The protein precipitated by TCA method was dissolved in sterile distilled water and was desalted. Antibacterial activity of the precipitate protein and the supernatant was checked. A clear zone of 21mm was noted around the well loaded with the protein sample against *Vibrio* sp.1. No zone of clearance was seen around the well loaded with the supernatant (Table 7).

Table.2 Antibiotic sensitivity pattern of bacterial pathogens

Bacterial pathogens	Co-Trimaxazole	Gentamycin	Amikacin	Bacitracin	Oxy Tetrcycline
Staphylococcus aureus	16	15	24	-	-
Vibrio sp.1	23	17	21	-	13
Salmonella typhi	11	18	22	-	17
Vibrio sp.2	17	20	21	-	10
Escherichia coli	9	15	23	-	-
Vibrio sp.3	16	18	21	9	7
Klebsiella pneumoniae	17	20	22	-	16
Enterococcus faecalis	15	-	-	-	-
Proteus vulgaris	14	17	25	-	-

Antibacterial activity of the protein precipitated by Ethanol-choloform method

The protein precipitated by Ethanol and choloform method was dissolved in sterile distilled water and was desalted. Antibacterial activity of the precipitate protein and the supernatant was checked. A clear zone of 23 mm was noted around the well loaded with the protein sample against *Vibrio* sp.1. No zone of clearance was seen around the well loaded with the supernatant (Table 8).

Plant pathogenic bacterial strains were used in both the agar-diffusion method and the agar-spot assay to evaluate the antagonistic activity of *P. syringae ciccaronei* and the spectrum of antimicrobial activity of the bacteriocin. Growth of *Ps. Syringae persicae* NCPPB2761 was inhibited by a *P. syringae ciccaronei* colony and its culture filtrate with the zone of inhibition 7mm (Lavermicocca *et al.,* 1999). Similarly the culture filtrate of *Pseudomonas aeruginosa* inhibited the growth of MRSA with the zone of inhibition 12mm.

The active metabolites produced by *Lactobacillus sp* NRRL B-227 exhibited various degrees of activities against *Staphylococcus aureus* with 21mm zone of inhibition (Atta *et al.,* 2009).

Table.3 Zone of inhibition of marine isolate against bacterial pathogens by point inoculation method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	-
2	*Vibrio* sp.1	35
3	*Salmonella typhi*	25
4	*Vibrio* sp.2	25
5	*Escherichia coli*	-
6	*Vibrio* sp.3	15
7	*Klebsiella pneumoniae*	12
8	*Enterococcus faecalis*	-
9	*Proteus vulgaris*	15

Table.4 Zone of inhibition of marine isolate against bacterial pathogens by agar well diffusion method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	14
2	*Vibrio* sp.1	17
3	*Salmonella typhi*	12
4	*Vibrio* sp.2	14
5	*Escherichia coli*	15
6	*Vibrio* sp.3	15
7	*Klebsiella pneumoniae*	12
8	*Enterococcus faecalis*	12
9	*Proteus vulgaris*	11

Table. 5 Zone of inhibition of supernatant of marine isolate against bacterial pathogens by agar well diffusion method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	20
2	*Vibrio* sp.1	25
3	*Salmonella typhi*	23
4	*Vibrio* sp.2	12
5	*Escherichia coli*	21
6	*Vibrio* sp.3	18
7	*Klebsiella pneumoniae*	14
8	*Enterococcus faecalis*	20

Table.6 Zone of inhibition of protein extract of marine isolate (ammonium sulphate) against bacterial pathogens by agar well diffusion method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	16
2	*Vibrio* sp.1	9
3	*Salmonella typhi*	11
4	*Vibrio* sp.2	9
5	*Escherichia coli*	-
6	*Vibrio* sp.3	-
7	*Klebsiella pneumoniae*	11
8	*Enterococcus faecalis*	12
9	*Proteus vulgaris*	12

Table.7 Zone of inhibition of protein extract of marine isolate (tca) against bacterial pathogens by agar well diffusion method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	-
2	*Vibrio* sp.1	21
3	*Salmonella typhi*	-
4	*Vibrio* sp.2	11
5	*Escherichia coli*	-
6	*Vibrio* sp.3	19
7	*Klebsiella pneumoniae*	11
8	*Enterococcus faecalis*	12
9	*Proteus vulgaris*	23

Table.8 Zone of inhibition of protein extract of marine isolate (ethanol) against bacterial pathogens by agar well diffusion method

S.No	Bacterial pathogens	Marine Isolate of *P.aeruginosa* (mm)
1	*Staphylococcus aureus*	-
2	*Vibrio* sp.1	23
3	*Salmonella typhi*	-
4	*Vibrio* sp.2	9
5	*Escherichia coli*	13
6	*Vibrio* sp.3	13
7	*Klebsiella pneumoniae*	20
8	*Enterococcus faecalis*	16
9	*Proteus vulgaris*	14

Similarly the active metabolite of *Pseudomonas aeruginosa* inhibited the growth of MRSA with the zone of inhibition 12mm. Using 16S r DNA sequencing the phylogenetic analysis revealed that all bioactive isolates belonged to the genus *Bacillus*. The isolates A184, A190 and A202 showed greater than 98.0% sequence similarity to *Bacillus subtilis* strain 168, while the isolate A 586 showed greater than 98.9% sequence similarity to *Bacillus pumilus* strain OM-F6. (Christian *et al.,* 2003).

Phylogenetic analysis using 16S rDNA sequences and the neighbour-joining method showed the X153 strain is a member of *Pseudoalteromonas* close to *P. piscicida, P. peptidysin,* and *Pseudoalteromonas* sp. named Y. this indicates that X153 strain is very close to a strain *Pseudoalteromonas* sp. Y, which displayed a algicidal effect against harmful micro-algae (Lovejoy *et al.,* 1998).

In this present study the organism was identified to be *Pseudomonas aeruginosa* by staining and biochemical tests. The peptide antibiotic produced by *L. plantarum* NRRL B- 227 was treated with Ammonium sulphate 60% saturation. The mixture were stirred for 2 hrs at 4°C and later centrifuged at 10,000 rpm for 50 min at 4°C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0. Dialysis was carried out against the same buffer for 18 hrs in spectrapor dialysis tubing. Assay of the antibacterial activity was carried out increase of antibacterial activity comparing with culture supernatant. (Atta *et al.,* 2009). In the present study the peptide antibiotic produced by *Pseudomonas aeruginosa* was treated with ammonium sulphate 30% saturation and the precipitate was desalted with desalting column. The fractions collected showed antibacterial activity against *Vibrio* sp.

Pseudomonas syringae ciccaronei bacteriocin was purified by Ammonium sulphate precipitation, and the molecular weight of bacteriocin was found to be approximately 100 kDa; this was confirmed by the data from gel filtration through Sephadex G150. However, a sample of bacteriocin purified using preparative gel electrophoresis revealed three bands on SDS-PAGE with molecular weights of 76, 63 and 45 kDa, respectively (Lavermicocca *et al.,* 1999).

The molecular mass of cerein 8A is quite distinct of other bacteriocins or BLS produced by *B. cereus* (Naclerio *et al.*. 1993; Oscariz *et al.,* 1999; Torak and Matijasic 2003). Also, these characteristics are contrasting with the antibiotics, which are cationic peptides with low- MW when estimated by SDS-PAGE (Montville and Chen 1998).

The antimicrobial peptide produced by *Pseudomonas* sp. 4B was purified by sequential precipitation, gel filtration, and ion-exchange chromatography process. A major peptide band of about 30 kDa was observed by SDS-PAGE analysis, coinciding with antimicrobial activity. This molecular mass is close to the lectin-like bacteriocin LlpA from a *Pseudomonas* sp. isolated from banana rhizosphere (Parret *et al.,* 2004). Similarly in the present work the molecular weight of the antimicrobial peptide (protein) from *Pseudomonas aeruginosa* was identified as 30 kDa.

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