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A STUDY ON THE PREVALENCE OF MULTIPLE DRUG RESISTANT *PSEUDOMONAS AERUGINOSA* (MDRPA) AMONG CLINICAL ISOLATES AND EFFICACY OF WATER EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST MDRPA

JAYACHANDRAN VP[1*], CHAYANI N[3], NAIR S[4] AND APPUKUTTAN G[2]

1, 4: Microbiology Research Unit, Pushpagiri Research Centre, Pushpagiri Institute of Medical Sciences, Tiruvalla, Kerala -689 101

2: Plant tissue culture and Molecular biology Lab, Department of Botany, University of Kerala,Kariavattom, Thiruvananthapuram-695 581

3: Department of Microbiology, Sri Ram Chandra Bhanj Medical College and Hospital, Cuttack, Orissa

*** Corresponding Author: Email: jaiwhy@gmail.com; Mob.: +91-9847823430**

ABSTRACT

Pseudomonas aeruginosa is an epitome of nosocomial agents and efficacious antimicrobial options are limited in treating *Pseudomonas* mediated infections.*P.aeruginosa* has special ability to resist antibiotics. Multiple drug resistant *P. aeruginosa* (MDRPA) is defined as isolates intermediate or resistant to at least three drugs in antipseudomonal cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones. Scientific community is in search of novel drugs against this bacteria. Medicinal plants are sources of potent antimicrobial agents. So prevalence of MDRPA among clinical isolates and evaluation of medicinal plant extracts as antipseudomonal agents hold prime significance. *P. aeruginosa* isolates were obtained from clinical conditions and their resistance to thirteen antibiotics were checked. Water extracts of nine medicinal plants were obtained by decoction method. The antibacterial efficacy of plant extracts was checked by well diffusion and minimum inhibitory concentration (MIC) was determined by microbroth tube dilution. 63.46% isolates were found to be MDRPA. Minimum resistance was extended to

imipenem followed by gatifloxacin and amikacin. Among the water extracts evaluated, the extracts of *Phyllanthus emblica* and *Aegle marmelos* were found to be effective against MDRPA with MIC of 200 μg/ml and 250 μg/ml respectively. MDRPA is prevalent among clinical isolates. Imipenem is the effective drug of choice but strains are developing resistance to them. The water extracts of *P. emblica* and *A. marmelos* can be used in the development of efficacious antipseudomonal agents.

Keywords: Multiple Drug Resistant *P. aeruginosa* (MDRPA), *Phyllanthus emblica, A. marmelos*

INTRODUCTION

According to the National Nosocomial Infections Surveillance (NNIS) System, *Pseudomonas aeruginosa* is among the leading pathogens causing nosocomial infections **[1]**. Infections caused by *P. aeruginosa* is associated with highest patient mortality rate and are difficult to eradicate from infected tissues or blood because these bacteria possess virulence and have limited susceptibility to antimicrobials **[2]**. An example of the adaptive capacity of *P. aeruginosa* is the development of multiple drug resistance (MDR), which creates an increasing number of difficult therapeutic problems **[3]**. The definition of multi drug resistance is not standardized in many of the studies published on this topic. Different agents within antimicrobial classes are selected as standards for resistance within each class, and the number of agents required for a strain to be classified as MDRPA is not always specified within these studies. MDRPA strain was defined as an isolate intermediate or resistant to at least three drugs of the following classes: β-lactams-cephalosporins (ceftazidime, cefepime), carbapenem- (imipenem), aminoglycosides (gentamicin, tobramycin, amikacin), and fluoroquinolones (ciprofloxacin) **[4]**. For the purpose this study along with the afore mentioned antibiotics resistance/intermediate to netilmycin, ofloxacin, levofloxacin and gatifloxacin are also being considered for the definition of MDRPA. To ensure optimal efficiency of antibiotic treatment against this species, antibiotic susceptibility tests must be interpreted with caution. It is essential to characterize the antibiotic resistance expressed by isolates. Particular resistance mechanisms may be suspected when the bacterium is resistant to several antibiotics in the same family **[5]**. Scientific community is in search of a novel and effective drug against this opportunistic bacterial pathogen.

For over 100 years medicinal plants have served as the models for a large percentage of prescription drugs. Many of these clinically proven drugs were initially used in the form of a crude extract in traditional systems of medicine for purposes that suggested potentially useful biological activity. Medicinal plants are now being re-assessed as models for antimicrobial agents **[6]**. Mainstream medicine has become more receptive to the research and development of antimicrobial agents and other plant-based medicines. This is due to the fact that typical antibiotics, products of microorganisms or their synthetic derivatives, have become ineffective, particularly as new viral, diseases remain untreatable with this type of drug. In addition, while pharmaceutical companies usually produce two or three new antibiotics on average per year, over the past twenty years the number of new antibiotics in the research and development pipeline has begun to decline. Thus, pharmaceutical companies have become increasingly interested in the potential use of antimicrobials and other drugs from plants **[7]**. In addition, the general public has become more aware of the overuse and misuse of antibiotics. The consumer is now very interested in alternative medicines, including medicinal plants as they are perceived as being both safe and effective **[8]**. In Ayurveda, the Indian system of medicine, many of the formulations are in the form of decoction.

Here in the present study was initiated to evaluate the prevalence of multiple drug resistance among *P. aeruginosa* isolates from different clinical conditions and the anti-pseudomonal efficacy of water extracts of selected phyto ingredients used in Indian traditional medicine.

MATERIALS AND METHODS

Plant Material and Extraction

Ripened fruits of *Phyllanthus emblica* Linn, *Garcinia cambogia* Gaertn. Desr, *Myristica fragrans* Houtt, leaves of *Cinnamomum tamala* Nees & Eberm, *Pimenta dioica* (L.), *Piper longum* L.and roots of *Aegle marmolos* (L.), *Premna latifolia* Roxb. and *Ixora coccinea* Linn were collected from the Sabarimala forests, Pathanamthitta district, Kerala, India. The plant species were confirmed using a referral herbaria and a voucher specimen was preserved in the Department of Botany, University of Kerala, Kariyavattam Campus, Tiruvananthapuram. The plant parts, free of diseases were cut into pieces and dried under shade. After powdering 40 g of the powder was soaked, boiled in 400 mL distilled water for three hours and the decoction was filtered, centrifuged and dried under reduced pressure

in a rotary evaporator to obtain a residue of crude extract. This crude extract was used for the antimicrobial study.

Isolation and Identification

During March to August 2011, 128 *Pseudomonas isolates* were obtained at the Department of Microbiology SCB, Medical College Hospital, Cuttack, Orissa. Out of 128 *Pseudomonas* isolates, 104 were confirmed as *P. aeruginosa.* The isolates were obtained from clinical conditions like burns, chronic obstructive pulmonary disease (COPD), surgery, skin infection, wound infection, eye and ear infections and urinary catheter and UTI. The specimens included blood, sputum, pus, urine, ear and throat swabs. Immediately after collection, specimen was spread on 5% blood, Mac Conkey and cetrimide agar plates and were incubated at 37°C for 18 h. Identification was done based on colony characteristics, pyocyanin production (bluish green pigment), gram staining, motility, oxidase, indole, methyl red, Voges Proskauer, citrate utilization tests and growth in Hugh Leifson medium and cetrimide agar.

Sterility Checking of the Water Extracts

The water extract of the plant materials (100mg each) was dissolved in 1ml sterile distilled water and loopful of these solutions were streaked on nutrient agar and Sabouraud dextrose agar (SDA) plates. NA plate were incubated at 37^{o}C for 48 hrs and the SDA plates were incubated at 28 ^{o}C for four days. These plates were checked for the appearance of colonies to confirm the absence of bacterial and fungal contaminants in the extracts.

Evaluation of Antibiotic Sensitivity by Disc Diffusion Method

18hrs broth cultures (0.5Mc Farland) were swabbed on Mueller Hinton agar plates and standard antibiotic discs (Himedia Ltd., Mumbai, India) of known potencies were placed on the media at equidistance. Antibiotic sensitivity was determined using Kirby-Bauer method as per CLSI guidelines **[9]**. The antibiotic discs included the aminoglycoside*:* gentamicin (G)10 µg, netilmycin (Nt) 30 µg tobramycin 10 µg and amikacin (Ak) 30 µg β lactam; a) cephalosporins – cephalexin (Ce) 30 µg, cefuroxime (Cu) 30 µg, ceftazidime (Ca) 30 µg, cefipime (Cpm) 30 µg, and carbepenem - imepenem (I) 10 µg and the fluoroquinolones *:* ofloxacin(Of) 10µg, ciprofloxacin (Cf) 10 µg, levofloxacin (Le) 5 µg, gatifloxacin (Gf) 5 µg,. The antibiogram was ascertained by measuring the diameter of zone of inhibition on 24 hrs incubation at 37^{o}C. The isolates were classified as sensitive/intermediate/ resistant. *P. aeruginosa* ATCC 27853 was used as control strain.

Evaluation of Antibacterial Activity of Plant Extracts

Antibacterial activity of the extracts was evaluated by agar well diffusion. 18hrs nutrient broth cultures of two MDR isolates were evaluated in the study. 100 mg of the water extracts were thoroughly mixed in 100% dimethyl sulfoxide (DMSO). Wells of standard size (6mm) were incised at specified distances in Mueller-Hinton agar and the broth cultures were swabbed on separate agar plates. 0.1 ml of the extracts were added into separate wells. Also 0.1ml of ciprofloxacin and imipenem at 100µg/ml concentration and amikacin and gentamicin at 300µg/ml concentration was loaded into wells and 0.1 ml DMSO served as control. After incubation at 37^{o}C for 24 hrs, diameter of zone of inhibition was measured. *P. aeruginosa* ATCC 27853 was used as control strain.

Determination of Minimum Inhibitory Concentration (MIC)

18 hrs Mueller Hinton broth culture of two isolates were selected for the evaluation of MIC. Assay was performed in 96-well microtitre plates. Extracts were dissolved in DMSO and diluted with Mueller Hinton broth to a concentration of 10mg/ml - 1mg/ml. Further 1:2 serial dilutions were performed by addition of culture broth to reach particular concentrations. Imipenem was also serially diluted with broth. Inoculum density of the test organisms was adjusted to that of 0.5 Mc Farland standard (10 µL., 1x 10^{8} CFU/ml). Broth was dispensed into wells of micro-titre plate followed by addition of the respective water extract and inoculum. Extracts (reconstituted in DMSO) were serially diluted into each of the wells. Total volume of the assay system in each well was kept 200 µL. A DMSO control was included in all assays. Plates were incubated at 37 ºC for 16-20 h and read at 600 nm in a plate reader (BIORAD 680). MIC was recorded as the lowest concentration at which no growth was observed. Triplicates were done.

RESULTS

The result of percentage resistance exhibited by *P. aeruginosa* isolates is summarized in **Figure 1**. Cephalexin (Ce)- 100% = cefuroxime (Cu) 100%, > ceftazidime (Ca) 84.6 % >, gentamicin(G) – 77.8% > cefipime (Cpm) – 65.3% > tobramycin(Tb) 62.5% > netromycin(Nt) 60.5% > ciprofloxacin(Cf) 55.7 % > ofloxacin (Of) 5l.9%, >levofloxacin (Le) 44.2%, >amikacin(Ak) 40.3%> gatifloxacin- (Gf) 32.6%> imepenem (I) - 26.9%, 63.5 % isolates extended resistance to three or more antibiotic classes excluding the first and second cephalosporins.

It was noted that out of the 104 isolates obtained 66 (63.46%) were MDRPA and the

Figure 2 shows the incidence of MDRPA and non MDRPA among the clinical isolates of *P. aeruginosa.*

Even after four days of incubation**.** no colonies were seen on both NA and SDA plates streaked with the different plant extracts. Two isolates were selected for evaluating the antibacterial activity of extracts i.e., an isolate showed resistance to all the antibiotics (Ps.1) and an isolate showed resistance to all antibiotics except imepenem (Ps.2).

In the well diffusion method zone of growth inhibition was observed around wells loaded with the water extract of *P.emblica* and *A. marmolos* as well as imipenem in case of Ps2 and *P.aeruginosa* ATCC 27853, where as zone of growth inhibition was observed around wells loaded with the water extract of *P.emblica* and *A. marmolos* in case of Ps1. No zone of inhibition was seen around wells loaded with the *G. cambogia*, *M. fragrans, C. tamala, P. dioica , P. longum, P. latifolia* and *I. coccinea* extracts in case of all the bacteria. The result of the well diffusion method is showed in **Table 1**.

The MIC of the aqueous extract of *P. emblica* and *A. marmolos*, was evaluated by micro tube broth dilution and the average value for MIC was found to be 200 µg/ml

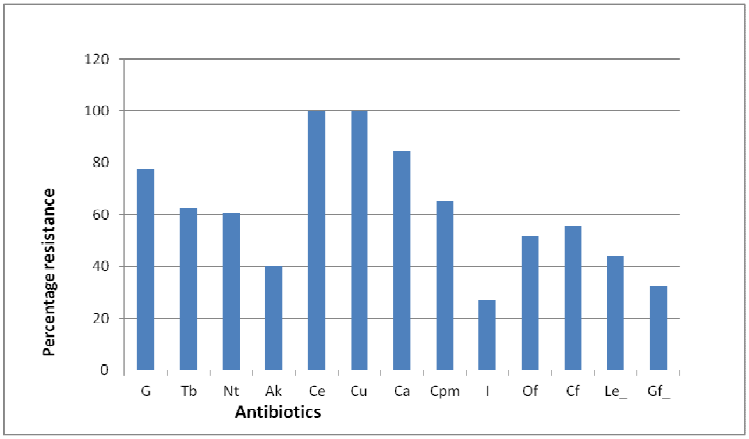

Figure 1: Percentage Antibiotic Resistance of Clinical Isolates of *P. aeruginosa*

Antibiotics evaluated : Gentamicin (G), Tobramycin (Tb), Netromycin (Nt), Amikacin (Ak), Cephalexin (Ce), Cefuroxime (Cu), Ceftazidime (Ca), Cefipime, (Cpm), Imepenem (I), Ciprofloxacin (Cf), Ofloxacin (Of), Levofloxacin(Le) and Gatifloxacin(Gf)

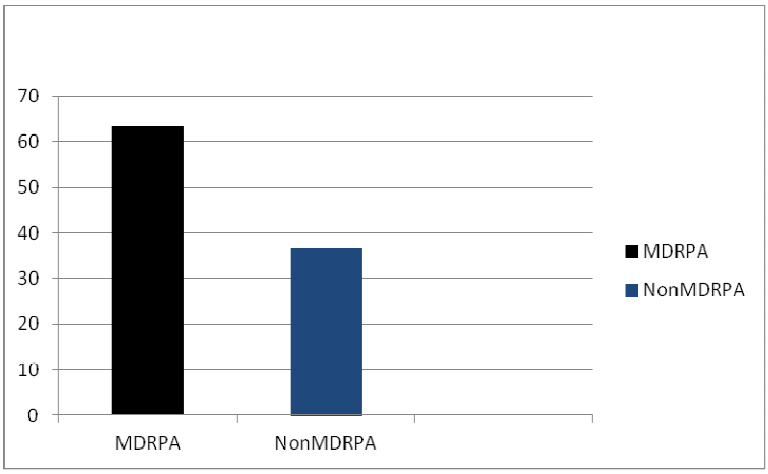

Figure 2: Percentage Incidence of MDRPA Among the Clinical Isolates of *P.Aeruginosa* Obtained During January To August 2011

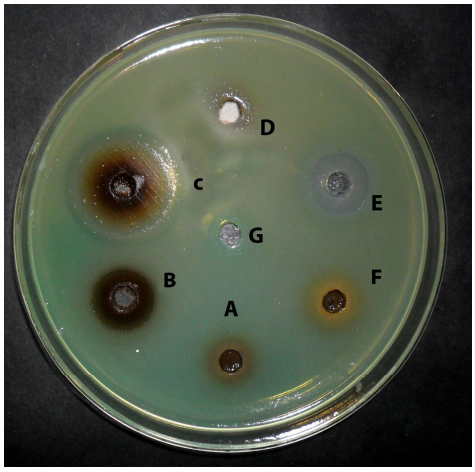

Figure 3: Zone of Growth Inhibition Around Wells Loaded With Plant Extracts and Antibiotic Solutions

A. *I. coccinea,* B. *A.marmolos*, C. *P.emblica*, D. *P. dioica,* E. imipenem, F. *P.latifolia* G. ciprofloxacin

Table 1: Well Diffusion Method: Diameter of Zone of Inhibition Around Well Loaded With Extracts and Imipenem

S. No.	Isolate	Diameter of zone of inhibition		
		E. officinalis	*A.marmolos*	Imipenem
1	*Ps 1*	21	19	---
2	*Ps 2*	23	18	17
3	*Paeruginosa aeruginosa 27853*	22	20	17

DISCUSSION

Bacterial resistance to antibiotics continues to be a significant problem as organisms appear to develop resistance to new drugs as rapidly as they are introduced. Antimicrobial agents with reliable antipseudomonal activity that are commonly prescribed are limited to only a few agents in three major pharmacological classes; β-lactams, fluoroquinolones (FQs) and aminoglycosides **[10]**. In the present study it was observed that 100% isolates were resistant to the first (cephalexin) and second (cefuroxime) generation cephalosporins. The isolates showed sensitivity to the third generation cephalosporin ceftazidime and fourth generation cefipime in an ascending order when turned from third to fourth generations and this high degree of resistance to the extended-spectrum cephalosporins viz ceftazidime and cefepime highlights the ability to produce an acquired extended-spectrum β-lactamase (ESBL) **[11]**.

All the aminoglycoside antibiotics except amikacin did not extend considerable activity. Forty isolates were resistant to amikacin, the present drug of choice. This study points to the descending efficacy of amikacin as an antipseudomonal agent. Imipenem was most effective, followed by gatifloxacin and amikacin. Almost 27% of isolates were resistant to imipenem belonging to the carbapenem group, known for their broad spectral activity and stability to hydrolysis by most β-lactamases. The carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant gram-negative bacilli, especially extended spectrum b-lactamase (ESBL) producers **[12]** and the present study tells the emergence of carbapenemase producing/ other associated virulent factors carrying strains among these isolates. Resistance extended to fluoroquinolones as ciprofloxacin could be associated to its wide spread prescribing practice.

In a study, from 2009 to 2010 by **Deepak *et al*** **[13]** with 193 *P. aeruginosa* isolates showed73% resistance to ciprofloxacin, 63% isolates were ceftazidime resistant and 3.7% isolates were resistant to imipenem . In the present study resistance to ciprofloxacin was comparatively lesser ie; 55.7% whereas 84.6% were ceftazidime resistant, also impenem resistance was significantly more ie 26.9% compared to the study by **Deepak *et al*** which underscored the emergence of carbapenem resistance among the isolates. In our study 63.46% isolates were identified as MDRPA. A study conducted in 2005 by **[14]** reported a prevalence rate of 44% of MDRPA **[14]** but in a study in 2007 conducted by **Jayakumar *et al*** in Tamilnadu only 22 %

isolates were MDRPA **[15]**. The present study invariably proved that MDR among *P.aeruginosa* isolates is increasing as there is a drastic increase in MDRPA percentage in our study compared to the mentioned reports.

The antipseudomonal β lactams - such as ceftazidime, cefepime and the carbapenems represent a major weapon against *Pseudomonas* infections, either for monotherapy/ for combination therapy, for which β-lactams almost invariably represent one of the components.**[10, 16]** Since their introduction ,carbapenems have been among the most powerful antibiotics for treating serious infections caused by Gram-negative nosocomial pathogens, including *Pseudomonas aeruginosa*. The emergence of strains containing β lactamases with carbapenem-hydrolyzing activity is of major clinical concern **[17]**.

Therefore, acquired resistance to these agents constitutes a major challenge for antpseudomonal chemotherapy, especially when it is associated with resistance to other classes of drugs viz., aminoglycosides/ fluoroquinolones.**[18]** Also 63.5% of isolates were resistant to at least 3 antibiotics making them MDRPA.. Reported mortality rates in adults with MDRPA range from 20% to 70%, depending on patient- and infection related factors **[19]**. It was showed that antimicrobial resistance, especially to ceftazidime or imipenem, adversely affected outcome in patients with *P. aeruginosa* bacteremia **[20]**.

Plant based antimicrobial compounds have great therapeutic potential due to lesser side effects as compared with synthe tic drugs, also little chance of developmen t of resistance. Plants are known to produc e secondary metabolites which are naturall y toxic to bacteria or inhibit their enzymes **[21]**. In certain pathophysiological conditions such as pregnancy the administration of antibiotics is harmful. Several studies point to the adverse effects of antibiotics and risk chances in pre pubertal patients discourages the use of many antibiotics **[22, 23, 24]** thereby justifying the search for antipseudomonal agents in plant resources.

The fruit of *P.emblica*, commonly known as amla is highly valued in traditional Indian medicine and it stregnthens nervous system, bone marrow and sense organs and it is used the treatment of diabetes and haemorrhagic diseases and useful in skin diseases **[25]**. Studies suggest the antidiabetic and antioxidant activities of *A marmolos* **[26]**. A thorough literature survey showed that our study is the pioneering one in the antibacterial activity of *P.emblica* and *A marmolos* against

MDRPA. The antipseudomonal efficacy of this plant is noteworthy as this is used in the preparation of many ayurvedic formulations. Hence alternatives to antipseudomonal agents can be researched.

A thorough literature survey showed that our study is the pioneering one in the antibacterial activity of these plant extracts against MDRPA from clinical cases.

Since the hot water extract showed the antibacterial activity, it can be deduced that antibacterial principles in these extracts are water soluble and are heat resistant, underlining the retention of efficacy during extraction procedures, ease of preparation and administration. Hence the activity of these plant extracts bear prime significance from the point of innovation in drug development that can be utilized as alternatives to the available antipseudomonal agents.

CONCLUSION

This study invariably proved that MDRPA is prevalent among clinical isolates and there is an increase in the incidence of MDRPA compared to the previous years. Eventhough resistance to imipenem is increasing it is the effective drug of choice. Resistance to cephalosporins underline the prevalence of β lactamase gene pool among these isolates. Amikacin was an effective antipseudomonal agent in the past years, this result highlights the importance of controlling the use of this antibiotic in hospitals because of the emerging resistance. The resistance exhibited to fluoroquinolones draws special interest as previous reports emphasized on the association of fluoroquinolone resistance and cross resistance to other antipseudomonal agents in clinical use. Evidently interpretive analysis of antibiotic susceptibility tests is essential for a satisfactory understanding of the action of antibacterial agents.

The search for new antimicrobial agents against MDR bacteria is a continuing process. Hence evaluation of safer plant based extracts/formulations for the development of antibacterial principles against MDRPA holds special connotation and underlines the utilization of alternative trials using standardization and clinical studies. The crucial rationale is to proffer suitable and competent antimicrobial drugs to the patient.

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