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Antibacterial Activity of Certain Medicinal Plants against Multiple Antibiotic Resistant *Pseudomonas aeruginosa* (MARPA) Isolated from Clinical Samples

T. M. Vijayalakshmi[1]* and R. Murali[2]

[1]Department of Medical Biochemistry, University of Madras, Taramani Campus, Chennai - 600 113, Tamil Nadu, India
[2]Department of Botany, Government Arts College for Men, Chennai - 600 035, Tamil Nadu, India

**Corresponding author*

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A B S T R A C T

In the present study 100 samples were collected for the identification of *Pseudomonas aeruginosa* isolates. Among 100 samples, Clinical specimens such as Urine (15 Samples), Pus swabs (surgical and non-surgical wounds) (25 samples), Stool (20 Samples), Ear swabs (25 samples) and Blood (15 Samples). Among the 100 clinical samples 44 isolates showed *Pseudomonas aeruginosa*. The bacterial isolates from the clinical specimens for the present study were confirmed as *Pseudomonas aeruginosa* by using conventional tests. The antibiotic resistant of *Pseudomonas aeruginosa* isolates showed a maximum of 69.6 percent to Gentamycin which was followed by other antibiotics. The leaves of selected plant species were collected and subjected to ethanolic extraction. The ethanolic leaves extract were tested against clinically isolated multiple antibiotic resistance *Pseudomonas aeruginosa* isolates. Antibacterial reference standard, Ciprofloxacin had equal effect on multiple antibiotic resistances *Pseudomonas aeruginosa* isolates from clinical isolates and Standard *Pseudomonas aeruginosa*. Zone of inhibition of Ciprofloxacin was compared with standard strains. The antimicrobial activity of plant extract was higher in *Chrysanthemum odoratum* than the other leaves extract tested against test bacteria. There is a scope to use ethanolic extract of the leaves of *Chrysanthemum odoratum* against infections caused by multiple antibiotic resistance *Pseudomonas aeruginosa*.

Introduction

The Pseudomonads are a diverse bacterial group of established and emergent pathogens. Members of the genus are major agents of nosocomial and community acquired infections, being widely distributed in the hospital environment where they are particularly difficult to eradicate. However, despite abundant opportunities for spread, *Pseudomonas aeruginosa* rarely causes community acquired infections in immunocompetent patients (Delden and Iglewiski, 1998).

A direct consequence of this is that there is a rise in patients with impaired immune defenses thereby leading to an increase in nosocomial infections especially by Gram-negative organisms such as *Pseudomonas.* Such organisms may be found in the patient's own flora, or in damp environmental sites or hospital equipments and medicaments.

They exhibit natural resistance to many antibiotics and antiseptics in which they may survive for long periods, and may even multiply in the presence of minimal nutrients and have the ability to colonize traumatized skin (Richard *et al.*, 1994).

In recent years, drug resistant bacteria have given rise to several serious outbreaks of infections with many deaths. *Pseudomonas aeruginosa* is known to cause a wide spectrum of diseases. It can infect almost any external site or organ, and therefore can be isolated from various body fluids such as sputum, urine, wounds, and ear swabs and from blood (Hugbo and Olurinola, 1992).

Constant bacteriological monitoring of the pathogens isolated from clinical specimens from patients in special units is necessary to draw attention of clinicians and infection control specialists to their current antibiotic susceptibility pattern and how often specific pathogens are isolated. This will form the bedrock of appropriate surveillance studies in such settings that will lead to developing, implementing and monitoring the impact of interventions such as the evidence-based, mutually agreed guidelines for the empirical antimicrobial therapy of common pathogens, effective infection control and public health guidelines. With the appearance of recent reports on the criteria for characterization of pseudomonads, identification of most pseudomonads isolated from clinical specimens is now possible in the diagnostic laboratory.

Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including viral infections. Single and Poly herbal preparations have been used numerously throughout history for the treatment of various diseases. Many studies have been carried out to extract various natural products for screening antimicrobial activity but attention has not been focused intensively on studying the combinations of these products for their antimicrobial activity.

One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents (Shah, 2005). Traditional healers claim that some medicinal plants such as *bixa spp*. and *bidens spp*. are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases. They can also be a possible source for new potent antibiotics to which pathogen strains are not resistant (Fabricant and Farnsworth, 2001).

The present study aims to identify the antibiotic resistant profiles of *Pseudomonas aeruginosa* from the clinical specimens and subjected to antimicrobial activity by using medicinal plants. The present study includes isolating *Pseudomonas aeruginosa* from the clinical samples and to identify the most predominant prevalence of *Pseudomonas aeruginosa* in clinical samples. Then, identify the multiple antibiotic resistant profiles of *Pseudomonas aeruginosa* from the clinical isolates. To study the antimicrobial activity of ethanolic extracts of flower of selected plants against antibiotic resistant *Pseudomonas aeruginosa* from the clinical isolates.

Materials and Methods

Sample collection

Clinical specimens such as Urine (15 Samples), Pus swabs (surgical and non-surgical wounds) (25 samples), Stool (20 Samples), Ear swabs (25 samples) and Blood (15 Samples) specimens of patient, were collected aseptically by using sterile cotton swabs in the present study.

Stool and Urine specimens collected by using sterile container. Samples were collected from in and around clinical laboratories at Chennai, Tamil Nadu, India.

Sterile swabs were used to collect samples from Pus samples. The surface area of wound was sterilized by alcohol soaked cotton. The pus samples were collected with a sterile swab and were kept in Pseudomonas isolation medium in order to maintain the viability of samples. Each sample was labeled with the needed particulars such as name, age and chemotherapy if under taken and the sample was brought to laboratory within 6 hours.

Urine samples after collected 1.5% of boric acid was added for controlling the growth overgrowth.

Bacterial isolates and conventional phenotypic characterization of *Pseudomonas aeruginosa*

The clinical specimens were plating them on 5% Sheep Blood agar. The specimens were also plated on blood agar and Macconkey agar for the isolation of concomitant organisms along with Pseudomonas. *Pseudomonas* were identified on the basis of growth on Pseudomonas isolation medium. Extensive phenotypic and physiological characterization was carried out by the conventional tests devised by Gilardi *et al.*, (1970 a,b).

Identification to species

Pseudomonas strains were further identified to the species level by using conventional physiological tests devised by Gilardi *et al.*, (1970 a,b) which are based on carbohydrate fermentation using 1% solution of following sugars: glucose, mannitol, rabinose, raffinose, sorbitol, sucrose, lactose, trehalose and inulin; by pyruvate utilization in 1% pyruvate broth; arginine decarboxylation in Moellers decarboxylase broth; hippurate hydroloysis; motility test; pigment production detected on tryptic soya agar (TSA); gelatin liquefaction; starch hydrolysis using 2% starch and polysaccharide production. Ability to produce enzyme phosphatase by *Pseudomonas* species was also tested using phenolphthalein-phosphate agar. Haemolysin production was detected in the strains of *Pseudomonas aeruginosa* by culturing the isolates on blood agar using 5% human blood. A single colony isolate was inoculated into 5mL Todd-Hewitt broth and incubated overnight at 37° C which was then added as an inoculum of one drop with the help of pasteur pipette. All tests were incubated at 37°C and read at 24 hours and 7 days.

Production of fluorescent pigment was determined on Pseudomonas Agar by incubating inoculated slants overnight at 37 C and then examining them for fluorescence by means of ultraviolet light. Negative cultures were left at room temperature and reexamined daily for up to 1 week before they were considered finally negative. Strains that were not fluorescent, but that were thought to be of the fluorescent species, were later retested on Pseudomonas F Agar. Production of pyocyanine and pyorubrin pigments was determined on Pseudomonas Agar by incubating inoculated slants overnight at 37°C and then at room temperature for up to 1 week. When diffusible pigment was observed, the color was recorded and chloroform was

then added to extract pyocyanine. Red diffusible pigment was considered to be pyorubrin; chloroform-extractable (blue) pigment was considered to be pyocyanine. Strains that did not produce pyocyanine or pyorubrin were retested later on Pseudomonas Agar P and Tech Agar. Motility was observed microscopically by the for motility, flagella, fat, and Gram stains were prepared from the overnight growth on Pseudomonas Agar F. In the application of Liefson's method, for flagella staining, commercially available precleaned slides were used. Intracellular fat granules were stained by the method of Burdon; a smear of an overnight slant culture of B. megaterium was used as a positive control. The oxidase test was performed by Kovac's method.

Antibiotic Susceptibility Test

Antibiotic susceptibility testing of the clinical isolates along with the quality control strains were performed using BHI agar instead of Muller Hinton agar by disk diffusion method. Antibiotic susceptibility test was conducted by adopting Kirby-Bauer disc diffusion method. The cultures were streaked closely with swab on the medium in the form of lawn. In the plate containing antibiotics such as: Ceftazidime (30 µg/disc), Imipenem (10µg/disc), Amikacin (30µg/disc), Gentamycin (10 µg/disc), kanamycin(30 µg/disc), and Ofloxacin (30µg) discs were placed and incubated at 37°C following overnight incubation the culture was examined for areas no growth around the disc (Zone of inhibition).

Antimicrobial Activity

Selection of plants

Based on the literature, the following plants were selected, on the presence of different groups of phytochemical compounds that they possess and based on the availability of the plants.

Collection of selected plants

The leaves of the plants above listed were collected largely in polythene bags and immediately transported to the laboratory for processing.

Preparation of crude extract

The leaves of the plants collected were individually washed with tap water, blotted with filter paper and spread over news paper for air drying under shade. After complete dryness, the leaves of individual plants were powdered using a mixer grinder. A known quantity of leaf powder (50 g) of each plant was taken in a 250 ml conical flask and added with 100 ml of ethanol (85%). Ethanol was used for the extraction of phytochemicals because it has the ability to dissolve the phytochemical compounds like tannins, polyphenols, flavonols, terpenoids and alkaloids (Ivanovska *et al.*, 1996). The ethanol-leaf powder mixtures were kept at room temperature for 48 hours and rapidly stirred using glass rod every 8 hours. After 48 hours, the extract of each plant was filtered through Whatmann No.1 filter paper to exclude the leaf powder. Then each filtrate was kept in beaker on a water bath at 45ºC until the solvent gets evaporated. A greasy final material (Crude extract) obtained for each plant was transferred to screw cap bottles and stored under refrigerated condition till use.

Antimicrobial Assay

Standard Strains Used

Standard reference strains of Multi-drug resistant *Pseudomonas aeruginosa* obtained from the Microbial Type Culture Collection

(MTTC) of Institute of Microbial Technology (IMTECH), Chandigarh were used for the present study. This Standard reference strains were maintained by subculture method in Nutrient agar slants.

Kirby Bauer Disc Diffusion Technique

This technique was used to test the sensitivity of selected test organisms to the ethanolic leaf extracts individually and in combination as described above (Bauer *et al.,* 1966).

Preparation of antimicrobial disc using crude extracts

Discs of 5 mm in diameter from a sheet of filter paper were punched out, placed in petridishes allowing a distance of 2-4 mm between each disc and sterilized in a hot air oven at 160ºC for 1 hour. After allowing the disc to cool, 20 µl (0.02 ml) of each test solution was added on to each disc and then the discs were dried at 37ºC in an incubator for one hour (Cheesbrough, 1984). For control set, the discs were added with distilled water (200 ml) containing 5ml ethanol + 2 drops of emulsifier at 20µl/disc.

Preparation of plates

The Petri plates of 100mm diameter with nutrient agar were swabbed with broth culture of each test bacteria in separate plates by using sterile swab. Over this, prepared antimicrobial discs were placed under aseptic conditions. Three discs of each extract were placed in triangles. Control sets with a standard antibiotic Ciprofloxacin (30 µg/disc) were simultaneously maintained. Also the discs without plant extract(discs prepared using 200 ml distilled water + 5 ml ethanol + one or two drops of emulsifier) were also maintained as another set of control for each test organism. The plates were then incubated at 37ºC for 24hours and the zone of inhibition (IZ) was measured and recorded.

Results and Discussion

There were 100 samples collected in this present study for the identification of *Pseudomonas aeruginosa* isolates. Among 100 samples, Clinical specimens such as Urine (15 Samples), Pus swabs (surgical and non-surgical wounds) (25 samples), Stool (20 Samples), Ear swabs (25 samples) and Blood (15 Samples). Among the 100 Clinical samples 44 isolates showed Positive for *Pseudomonas aeruginosa* (Table.3). The highest isolates were obtained in Ear swab samples. Results on percentage of each samples showed positive for *Pseudomonas aeruginosa* isolates was tabulated in Table-3 and Chart.1.

The phenotypic characteristics of *Pseudomonas aeruginosa* isolates from clinical samples are indicated in Table 1 - 2. Preliminary identification of the isolates was performed by conventional tests (gram-negative bacilli, pigment production, Growth on cetrimide, growth at 45°C, growth in DCA, and TTC positive). Additional biochemical tests were performed as previously described to confirm speciation, including production of acid in the presence of Lactose, mannitol, fructose, glucose, mannose and sucrose (Table.2).

Out of 100 samples, 44 samples showed *Pseudomonas aeruginosa*. All isolates of *Pseudomonas aeruginosa* subjected to antimicrobial sensitivity test. The antibiotic resistant of *Pseudomonas aeruginosa* isolates showed a maximum of 69.9 percent to Gentamycin. Ethanolic extracts of the plant leaves includes *Asparagus racemosus*, *Acacia arabica*, *Chrysanthemum odoratum, and Cassia tora* showed antimicrobial activity against multiple antibiotic resistant *Pseudomonas aeruginosa* (Table.5).

Antibacterial reference standard, Vancomycin had equal effect on multiple antibiotic resistant *Pseudomonas aeruginosa* tested and also tested for standard *Pseudomonas aeruginosa* strains (Table.6).

Clinically significant infections with *P. aeruginosa* should not be treated with single antibiotic, due to that bacteria can rapidly develop resistance when such a single antibiotics are used. According to different reports, multiple drug resistances to *P. aeruginosa* are spreading hazards in the world and making the therapeutic management of these patients more problematic (McCallum *et al.,* 2001; Navon-Venezia *et al.,* 2005). An alternative way to combat the problem of microbial resistance is development of new antibacterial agents for substitution with ineffective ones. In plants, secondary products can have a variety of functions; some may have bearing on potential medicinal effects for humans.

Recently, different reports from different countries using different pathogens were published showing the antimicrobial activities of medicinal plants (Rios and Recio, 2005). Many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites (Briskin, 2000). While many medicinal plants exert their medicinal actions without serving a nutritional role in the human diet and may be used in response to specific health problems (Briskin, 2000). Results of this kind herald an interesting promise of constructing a potentially active antibacterial additive agent of plant origin.

The results showed the prevalence of *Pseudomonas aeruginosa* from 100 various clinical samples. About 33% (5 isolates) from urine, 43.4% (11 isolates) from pus swabs, 45% (9 isolates) of stool, 52% (13 isolates) of Ear swabs sample and 40.0% (6 isolates) of blood samples, *Pseudomonas aeruginosa* have been isolated and characterized.

This study is undertaken to reveal the emergence of Multiple antibiotic resistance of *Pseudomonas aeruginosa* which posing a serious therapeutic challenge. *Pseudomonas aeruginosa* was found to be most predominant in Ear swab samples rather than in other samples and least percentage of isolates have been observed in urine samples.

The parallel reports have been reported by Olayinka *et al.,* in the year 2004 from hospitalized patients. A total 92 *Pseudomonas aeruginosa* were identified and 19.6% of the isolates were resistant to three or more of the antibiotics tested, with the most prevalent resistance pattern being ceftazidime + gentamicin + perfloxacin + ofloxacin (27.8%).

Out of 100 samples, 44 samples showed *Pseudomonas aeruginosa*. All isolates of *Pseudomonas aeruginosa* subjected to antimicrobial sensitivity test. The highest isolates (13) were obtained in Ear swab samples.

It reveals the emergence of multiple antibiotic resistances among unusual species of *Pseudomonas aeruginosa* posing a serious therapeutic challenge. Precise identification of *Pseudomonas aeruginosa* to species level enables us to access the species-specific antimicrobial resistance characteristics, apart from knowing the epidemiological pattern and their clinical significance in human infections. This study was carried with an aim to isolate Multiple antibiotic resistant *Pseudomonas aeruginosa* from Clinical samples from Hospital laboratories and subjected to antibiotic sensitivity test by using Gentamycin, Kanamycin, Amikacin, Ofloxacin, Streptomycin, Ceftazidime.

Table.1 The lists of plants selected for the study are given here under (Figure1 to 4)

Plants selected	Family	Major class of phytochemical(s) present	References
Asparagus racemosus	Liliaceae	Terpenoid	**Duke (1985)**
Acacia arabica	Myrtaceae	Polyphenol	
Chrysanthemum odoratum	Asteraceae	Flavonol	
Cassia tora	**Myrtaceae**	**Polyphenol**	

Table.2 Phenotypic Characteristics of *Pseudomonas aeruginosa* Isolated from Clinical Samples.

Sl. No.	Phenotypic characters	Activity
1.	Gram Reaction	**Gram negative short rods**
2.	Motility	**motile**
3.	Growth on nutrient agar	**Green colour pigment colonies**
4.	Growth on blood agar	**β - hemolysis**
5.	Growth on SSAgar	**Colourless colonies formed**
6.	Growth on DC Agar	**Colourless colonies formed**
7.	Growth on MacConkey Agar	**Colourless colonies formed**
8.	Growth on TTC	**Growth occurs**
9.	Growth at 6.5%0NaCl	**Negative**
10.	Growth at 2.5% NaCl	**Growth occurs**
11.	Growth at pH5.6 (SGA)	**Growth occurs**
12.	Growth on cetrimide	**Green colour pigment colonies**
13.	Growthat42C	**Green colour pigment colonies**
14.	ONPG	**Netative**
15.	Hydrogen sulfide (KIA)	**Negative**
16.	Nitrite	**Negative**
17.	Arginine dihydrolase	**Positive**
18.	Lysine decarboxylase	**Negative**
19.	Ornithine decarboxylas	**Negative**
20.	Phenylalanine deaminase	**Negative**
21.	Catalase	**Positive**
22.	Oxidase	**Positive**
23.	IMViC	
	(i) Indole	**Positive**
	(ii) Methyl red	**Negative**
	(iii) Voges-Proskauer	**Negative**
	(iv) Citrate	**Positive**
24.	Gelatin liquefaction	**Positive**
25.	Lipase	**Positive**
26.	Caseinase	**Positive**
27.	Lecithinase	**Negative**
28.	Deoxyribonuclease	**Negative**
29.	**Starch hydrolysis**	**Negative**

ONPG, o-nitrophenylfl- D-galactopyranoside, KIA, Kligler Iron Agar; SS Agar, Salmonella Shigella Agar; DC Agar, Desoxycholate Agar; TTC, triphenyl tetrazolium chloride; SGA, Sabouraud's Glucose Agar; BMM, basal mineral medium

Table.3 Sugar Fermentation Test

S. No.	Sugars	Activity
1.	Glucose	**Positive**
2.	Lactose	**Positive**
3.	Mannitol	**Positive**
4.	Sucrose	**Negative**
5.	Maltose	**Negative**
6.	Fructose	**Positive**
7.	Mannose	**Positive**
8.	Inulin	**Negative**
9.	Rhaffnose	**Negative**
10.	Xylose	**Negative**
11.	Arabinose	**Negative**
12.	Inositol	**Negative**
13.	D-Trehalose	**Negative**
14.	**Malonate**	**Positive**

Table.4 Distribution of *Pseudomonas aeruginosa* from Various Clinical Specimens

S. No.	Clinical samples (n=100)	No. of *Pseudomonas aeruginosa* positive	Percentage of *Pseudomonas aeruginosa*
1	Urine (15 Samples)	5	**33.0**
2.	Pus swabs (25 samples)	11	**43.4**
3.	Stool (20 Samples)	9	**45.0**
4.	Ear swabs (25 Samples)	13	**52.0**
5.	**Blood (15 Samples)**	**6**	**40.0**

Table.5 Antibiotic Resistance Percentage of *Pseudomonas aeruginosa* Isolates from Clinical Samples (n=100)

Test Samples	No. of isolates	Antibiotics used					
		GM	KM	AM	OF	SM	CF
Urine	5	60.0(3)	60.0(3)	40.0(2)	20.0(1)	60.0(3)	40.0(2)
Pus swabs	11	72.7(8)	45.4(5)	45.4(5)	72.7(8)	54.4(6)	72.7(8)
Stool	9	44.4(4)	33.3(3)	44.4(4)	33.3(3)	22.2(2)	44.4(4)
Ear swabs	13	69.6(9)	61.2(8)	38.4	69.6(9)	69.6(9)	61.2(8)
Blood	6	66.6(4)	50.0(3)	66.6(4)	33.3(2)	50.0(3)	66.6(4)

The number of resistant isolates ().
GM-Gentamycin; KM-Kanamycin; AM-Amikacin; OF-Ofloxacin; SM-Streptomycin; CF- Ceftazidime

Table.6 Effect of Ethanolic Extracts of Selected Plant Leaves on Multiple Antibiotic Resistance *Pseudomonas aeruginosa*

S. No.	Ethanolic extract of the plant leaves used	Zone of inhibition in cm	
		IMARPA	SPA
1	*Asparagus racemosus*	1.8	**1.8**
2	*Acacia arabica*	1.4	**1.5**
3	*Chrysanthemum odoratum*	1.9	**2.0**
4	***Cassia tora***	**1.3**	**1.2**

(Values are mean of three replicates)
IMARPA – Clinically isolated multiple antibiotic resistance *Pseudomonas aeruginosa*
SPA – Standard *Pseudomonas aeruginosa*

The antibiotic resistant of *Pseudomonas aeruginosa* isolates showed a maximum of 69.6 percent to Gentamycin which was followed by other antibiotics in the following order: Kanamycin, Amikacin, Ofloxacin, Streptomycin, Ceftazidime (table.4).

Since the development of resistance to antibiotics by the pathogenic strains of *Pseudomonas aeruginosa* is an ever increasing problem, a suitable and possible alternate chemotherapeutic compounds which are of plant origin i.e., phytochemical compounds such as alkaloids, terpenoids, polyphenols, flavonoids and steroids are tried for effective means of controlling Multidrug resistant bacteria like *Pseudomonas aeruginosa.*

The present study provides evidence that can be used in subsequent risk assessment exercises to elucidate the role of clinical sample in the dissemination of antibiotic resistance *Pseudomonas aeruginosa* to human populations. This has lead to the emergence and dissemination of resistant bacteria and resistance genes in humans. The main sector of resistance-increasing medicine usage, in regard to human health, lies within the health care sector. Monitoring of antibiotic resistance, antibiotic resistance transfer, and antibiotic use and studies on the dissemination of antibiotic resistance in humans is essential to obtaining consistent and reliable data on the epidemiology of resistance of Enterococcal isolates from humans for the treatment of Enterococcal diseases. However, data such as those presented here offer evidence that should be helpful in the identification of future study topics and initiatives aimed at reducing the public health burden of antibiotic-resistant pathogens.

The result on the analysis of ethanolic extracts of the plant leaves includes *Asparagus racemosus, Acacia arabica*, *Chrysanthemum odoratum*, *and Cassia tora* showed antimicrobial activity against multiple antibiotic resistant clinical isolates *Pseudomonas aeruginosa and Standard strains of Pseudomonas aeruginosa* that include 1.9 and 2.0 respectively (Table.5). Among the plant leaves tested *Chrysanthemum odoratum* showed strong activity against multiple antibiotic resistant *Pseudomonas aeruginosa*. Ethanolic extracts of *Asparagus racemosus* showed moderate antimicrobial activity against antibiotic resistant *Pseudomonas aeruginosa* (Table.5). Antibacterial reference standard, Ciprofloxacin had an equal effect on multiple antibiotic resistant *Pseudomonas aeruginosa* tested (Table.6).

Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the

treatment of infectious diseases caused by resistant microbes. There is a possibility of using plant extracts against Multiple antibiotic resistance *Pseudomonas aeruginosa* has been observed from the results.

Minimizing and controlling antibiotic use in ICU patients should help reduce the risk of antimicrobial resistance in Pseudomonas bacteria. The extensive use of broad-spectrum antibiotics in the hospital can be probably responsible for the emergence and selection of resistant strain.

The antimicrobial activity of plant extract was higher in *Chrysanthemum odoratum* than the other leaves extract tested against test bacteria. There is a scope to use ethanolic extract of the leaves of *Chrysanthemum odoratum* against infections caused by multiple antibiotic resistance *Pseudomonas aeruginosa*.

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