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Evaluating Susceptibility of Environmental Isolates of Pseudomonas aeruginosa to Chlorhexidine Gluconate and Aqueous Extract of Peganum harmala

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Abstract

***Background and Objective*:** *Pseudomonas aeruginosa* is an important case of nosocomial infections and a major health problem. The increased emergence of resistance to antibiotics and disinfectants among these bacteria, necessitates the production of new antimicrobials with wider activity and low toxicity. This study was carried out to evaluate inhibitory effects of chlorhexidine gluconate and extract of *Peganum harmala* on multi-drug resistant (MDR) *P. aeruginosa*.

Material and Methods: 39 *P. aeruginosa* strains were isolated from 114 environmental samples. After identifying MDR strains, minimal inhibitory concentration (MIC) of chlorhexidine gluconate (20%) and *P. harmala* extract against the isolates was determined by broth microdilution method.

Results: Overall, 27 (69.2%) *P. aeruginosa* isolates resistant to quinolones, aminoglycoside, cephems and carbapenems were reported as MDR strains. The MIC of chlorhexidine gluconate was ≥ 1000µg/ml, which was 4-fold higher than the MIC of *P. harmala* extract (MIC = 500µg/ml). MIC of chlorhexidine gluconate and *P. harmala* extract against *P. aeruginosa* isolates differed significantly (P=0.01).

Conclusion: Compared to chlorhexidine gluconate, *P. harmala* extract has a higher antibacterial effect on MDR *P. aeruginosa* isolates from environment. Further research is required to verify the efficacy of this plant extract for disinfection of equipment in clinics and local kitchens.

Keywords: Pseudomonas aeruginosa [MeSH], Peganum [MeSH], Chlorhexidine gluconate [MeSH]

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Highlights

- Two biocides for control of environmental *P. aeruginosa* were identified.
- The aqueous extract of *P. harmala* had favorable antimicrobial activity against MDR isolates in comparison with chlorhexidine gluconate.
- *Peganum harmala* has low toxicity and is relatively cheap, which makes it useful in control of clinics and local kitchens surfaces.

Introduction

The prevalence of hospital-acquired infections has been increasing since 1980, particularly due to the widespread spread of multidrug-resistant (MDR) bacteria worldwide. These bacteria can spread easily in hospital settings. Therefore, hospital disinfection policies play an important role in controlling nosocomial infections (1). *Pseudomonas aeruginosa* is an opportunistic pathogen and one of the most important causative agents of nosocomial infections.

Intrinsic resistance to antibiotics and disinfectants is the most important factor that identifies this bacterium as pathogenic. This bacterium has a high resistance rate to a wide range of antimicrobials and antibiotics. Improper or incorrect use of anti-pseudomonal antibiotics has led to the development of MDR strains, which complicates the treatment process (2, 3).

Biocides, including antiseptics and disinfectants, are widely used in hospitals and other health settings for disinfection of various medical devices and surfaces. However, the recent increase in the rate of microbial resistance to biocides has raised some concerns, and current principles have not been very successful in controlling nosocomial infections by MDR pathogens (4).

There are currently several laboratory reports of the emergence of possible bacterial resistance to biocides, which have often been associated with exposure to lower concentrations of biocides. The reduced bacterial susceptibility to biocides and the possible association between biocide resistance and antibiotic resistance is a relatively new and important health concern. This phenomenon may lead to inadequate disinfection of environmental surfaces and increased spread of antibiotic- and disinfectant-resistant hospital pathogens. Chlorhexidine gluconate is a positively-charged biguanide biocide that interact with the negatively-charged surface of microbial cells, thus destroying the integrity of the cell membrane (5, 6).

Medicinal plants have been effectively used for treatment and prevention of infectious diseases. *Peganum harmala* seed is a perennial plant belonging to the *Zygophyllaceae* family that has long been considered as one of the most important medicinal plants in Iranian traditional medicine. Numerous pharmacological properties have been attributed to *P. harmala*, including antimicrobial and monoamine oxidase inhibitory effects (7). This plant grows in semi-arid regions and sandy soils, especially in North Africa and the Middle East region as well as in different parts of Iran including the, Golestan, Khorasan, Sistan and Baluchestan and Yazd provinces. The total content of pharmacologically active alkaloids in *P. harmala* seeds is 2-6% (8, 9). Amiri and Fozouni in 2020 studied the antibacterial effects of *P.harmala*. This research demonstrated that the aqueous extract of *P.harmala* had outstanding antimicrobial effects on the Drug-resistant *Acinetobacter baumannii* isolates (10). Given the importance of pathogens spread from the environment to humans, promoting knowledge of health care personnel about the prevalence of biocide-resistant isolates in a region and understanding optimal disinfection protocols for effective control of these pathogens seem necessary. This study was aimed to evaluate inhibitory effects of chlorhexidine gluconate and extract of *Peganum harmala* on environmental multi-drug resistant (MDR) *P. aeruginosa*.

Materials and Methods

- ***Bacteria isolation***

In this descriptive-analytical study, 114 samples were collected from equipment in six beauty clinics and local kitchens over a period of six months. After initial culture in blood agar and cetrimide agar (Merck, Germany) for 24 hours at 37 °C, *Pseudomonas* strains were identified by gram staining, oxidation fermentation in Triple Sugar Iron (TSI) agar, positive oxidase test, positive catalase test, pigmentation, positive motility, decarboxylation of arginine and growth at 42 °C.

- ***Determination of minimum inhibitory concentration of biocide***

In broth microdilution method, 20% chlorhexidine gluconate biocide (Unilab chemical, India) was dissolved in sterile distilled water to prepare a 2000 µg/ml stock solution. A suspension from MDR-*P. aeruginosa* equivalent to a half McFarland standard (1.5×10^8 CFU/ml) was prepared by reading absorbance 570 nm. The isolates were defined resistant to ciprofloxacin (CIP5), ceftazidime (CTZ10), amikacin (AN30), imipenem (IPM10) and aztreonam (ATM30) antibiotic disks (purchased from Padtan Teb Co., Iran) using Kerby-Bauer method according to the instructions of the Clinical and Laboratory Standards Institute. (CLSI,2020, 11). To determine the minimum inhibitory concentration (MIC), chlorhexidine gluconate in the range of 2000 to 7 µg /ml was inoculated to 96-well plate containing Müller Hinton Broth (Merck, Germany). Finally, the bacterial suspension equivalent to 0.5 McFarland standard was inoculated into the wells. Wells containing MHB and biocide and containing MHB and bacterial suspension were considered as negative and positive controls respectively. After overnight incubation at 37 °C, the lowest concentration at which no bacterial growth was observed was reported as the MIC.

- ***Plant collecting and preparation of P. harmala extracts***

P. hamala seeds were collected in around the city of Gorgan, "northern Iran" in April 2020. The seeds were washed, dried at room temperature and eventually were powdered with an electric mill. Extraction was done by maceration using ethanol as solvent. For this purpose, 25 g of dried seeds of *P. harmala* were mixed with equal amounts of heated-70% ethanol. The mixture was placed on a shaker for 3 to 4 days at room temperature. The resulting solution was filtered with a filter paper (Whatman No. 2, USA). To prepare the aqueous extract, 25 g of milled seeds were soaked in 250 ml of sterile distilled water for three days. The solution was filtered and the solvent was evaporated under a fume hood, and dry weight was measured and freeze-dried.

- ***determination of Peganum harmala extract MIC***

After preparing a stock solution from the extracts with 1% dimethyl sulfoxide, MIC of *P. harmala* extract at a range of 2000 to 7 µg/ml was determined by the broth microdilution method. Serial dilutions were made by adding 50λ of the extract to wells of a 96-well plate containing 50λ MHB. Then, 50λ of MDR- *P. aeruginosa* suspension (with turbidity of 0.5 McFarland) were separately inoculated into each well. After overnight incubation at 37 °C, the growth rate was measured and compared with that of the positive control (without the extract) and the negative control (without bacterial suspension). *Pseudomonas aeruginosa* ATCC 27853 was used as a control strain.

- ***Statistical Analysis***

Data were presented as mean ± standard deviation. All data were analyzed by SPSS software (version 23) using independent t-test, one-way analysis of variance and Fisher's exact test. All statistical analyses were performed at significance of 0.05.

Results

Of 39 (11.4%) *P. aeruginosa* isolates, 27 (69.2%) were determined as MDR. According to results, chlorhexidine gluconate and *P. harmala* extract inhibited growth of the MDR *P. aeruginosa* isolates in a dose-dependent manner and at concentrations of ≥1000 µg/ml and 500 µg/ml, respectively. MIC of chlorhexidine gluconate, which inhibited the growth of MDR *P. aeruginosa* isolates was 4-fold higher than the MIC of *P. harmala* extract (Tables 1 and 2)

Table 1. Distribution of MIC of chlorhexidine gluconate and *Peganum harmala* extract against MDR *Pseudomonas aeruginosa* isolates

MDR isolates	Organism Identification number	MIC ($\frac{\mu g}{ml}$)	
		PH	CHG
P. aeruginosa (n=27)	$P.a_{2}$	500	2000
	$P.a_{24}$	500	1000
	$P.a_{26}$	250	500
	$P.a_{27}$	62.5	500
	$P.a_{29}$	500	1000
	$P.a_{31}$	125	1000
	$P.a_{34}$	500	2000
	$P.a_{35}$	125	1000
	$P.a_{36}$	500	2000
	$P.a_{37}$	500	1000
	$P.a_{38}$	500	1000
	$P.a_{39}$	125	1000
	$P.a_{33}$	125	1000
	$P.a_{32}$	250	1000
	$P.a_{3}$	1000	2000
	$P.a_{5}$	250	1000
	$P.a_{6}$	250	2000
	$P.a_{8}$	250	1000
	$P.a_{11}$	500	1000
	$P.a_{12}$	125	1000
	$P.a_{14}$	250	1000
	$P.a_{16}$	500	1000
	$P.a_{18}$	1000	2000
	$P.a_{20}$	62.5	1000
	$P.a_{21}$	500	1000
	$P.a_{22}$	250	1000
	$P.a_{23}$	62.5	500

After performing the independence test, it was found that the two criteria of antibiotic classification and severity of effect are related to each other (P = 0.035). There was a significant difference (with mean and standard deviation (113.00±0.50) between the minimum concentration of biocides and the minimum concentration of *P. harmala* extract that inhibits the growth of *P. aeruginosa* isolates (P = 0.01).

Table 2. Mean MIC of chlorhexidine gluconate and *Peganum harmala* extract against MDR *Pseudomonas aeruginosa* isolates

MIC Range ($\frac{\mu g}{ml}$)	$\frac{P.aeruginosa}{CFU/ml}$	*P-value*
P. harmala	62.5-1000	0.02*
chlorhexidine gluconate	500-2000	0.053

p<0/05(*significant)

Discussion

Pseudomonas aeruginosa is a major cause of opportunistic nosocomial infections that are often severe, with a high mortality rate (12, 13). In the present study, the frequency of drug-resistant *P. aeruginosa* isolates was 34.2%, which is higher than the frequency reported in the same area in 2017 (12). In addition to sample type and time of study, factors such as infection control policies (prevention with biocides), the amount and method of antibiotic administration, study population, type of predominant strain and laboratory diagnosis methods could affect identification of drug-resistant strains (14).

Factors influencing the selection of disinfectant include effectiveness of, non-toxicity, skin compatibility, cost-effectiveness, odor, availability and ease of use. Chlorhexidine is an antiseptic that acts against a wide range of gram-positive and gram-negative bacteria, as well as some fungi and viruses. An advantage of this anti-septic compound is that it can bind to the surface of many substrates without losing its disinfecting activity and then released slowly, which results in long-term maintenance of its effectiveness in the environment (15, 16). It has been demonstrated that chlorhexidine gluconate has favorable antimicrobial activity against oral bacteria (17). Moreover, MIC concentrations of chlorhexidine gluconate could prevent biofilm formation in bacteria causing nosocomial infections, while sub-MIC concentrations of this compound can stimulate biofilm production (18).

A previous study reported that both chlorhexidine and chamomile extract had good inhibitory activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* isolates from patients in intensive care unit, but the antimicrobial effect of chlorhexidine was generally greater than that of the chamomile extract (19). In this study, *P. harmala* had a much better antibacterial effect compared to the chlorhexidine gluconate. Given the results obtained from our study, the aqueous extract of *P. harmala* has favorable antimicrobial activity against MDR *P. aeruginosa* isolates.

A previous study also demonstrated the favorable antimicrobial activity of this extract against various species of bacteria, particularly drug-resistant bacteria (20).

In our study, more than 90% of MDR *P. aeruginosa* isolates were susceptible to the aqueous extract of *P. harmala*, researchers stated that the aqueous extract of *P. harmala* is significantly more effective against *Lactobacillus* and *Candida* spp. compared to the aqueous extract (21).

Our results also show that the type of solvent has a significant impact on the extraction of active compounds from the plant. Polar solvents such as ethanol and water are thought to be more suitable for extraction of bioactive secondary metabolites of plants. The antibacterial activity of the ethanolic extract of *P. harmala* can be attributed

to the presence of alkaloids i.e. harmine and harmaline. The abundance of these compounds in the aqueous extract may also explain the higher antimicrobial activity (22). Researchers in Iran demonstrated that the antibacterial effect of aqueous extract of *P. harmala* was about 1.23 times higher than that of the ethanolic extract on *A. baumannii* clinical isolates (10).

In this study, the antibacterial activity of the aqueous extract increased in a dose-dependent manner in a way that 1000 µg/mL of the extract was able to inhibit the growth of almost all MDR *P. aeruginosa* isolates. Since the present study was conducted during the COVID-19 pandemic, the beauty clinics and local kitchens were either closed, or working part-time, which justifies the relatively low frequency of isolates.

Conclusion

We observed that the prevalence of MDR *P. aeruginosa* isolates is high in environment. Fortunately, the aqueous extract of *P. harmala* had favorable antimicrobial activity against these isolates in comparison of chlorhexidine. This extract has low toxicity and great antimicrobial activity, which makes it a cost-effective alternative to chemical antibiotics for the treatment of infections caused by drug-resistant *P. aeruginosa* strains.

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