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Antibacterial Effects of *Zataria multiflora*, *Ziziphus*, Chamomile and *Myrtus communis* Methanolic Extracts on IMP-Type Metallo-Beta-Lactamase-Producing *Pseudomonas aeruginosa*

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Abstract

Background: Carbapenem resistance due to acquired metallo-beta-lactamases (MBLs) is considered to be more serious than other resistance mechanisms.

Objectives: The aim of this study was to examine the effects of the methanolic extracts of *Zataria multiflora*, *Ziziphus*, Chamomile and *Myrtus communis* leaves on IMP-type MBL- producing *Pseudomonas aeruginosa* strains.

Materials and Methods: This cross-sectional descriptive study was conducted on burn patients hospitalized in Shahid Motahari Hospital, Tehran, Iran, during 2012 - 2013. Antibiotics and extracts susceptibility tests were performed using the disc diffusion and broth micro dilution methods. The metallo-beta-lactamase detection was performed by combination disk diffusion test. The bla (VIM) and bla (IMP) genes were detected by polymerase chain reaction (PCR) and sequencing methods.

Results: Eighty-three out of 96 samples were imipenem-resistant *P. aeruginosa* strains. Among 83 imipenem-resistant *P. aeruginosa* strains, 48 (57.9%) were MBL producers. Polymerase chain reaction and sequencing methods proved that these isolates were positive for blaIMP-1 genes, whereas none were positive for bla (VIM) genes. The minimum inhibitory concentration (MIC) for imipenem was 128 (μg/mL) for all strains. The MIC and minimum bactericidal concentration (MBC) of *M. communis* were 6.25 and 12.5 (mg/mL) for all isolates, respectively; the MIC and MBC of *Z. multiflora* were somehow the same. Methanolic extract of Chamomile showed to have a beneficial effect on this strain, while the *Ziziphus* leaves methanolic extract showed no significant effect on these isolates.

Conclusions: The results of this study reveal that the *M. communis* extract and methanolic extract of Chamomile have a high antibacterial effect on regular and IMP-producing *P. aeruginosa* strains; so, these extracts can be suitable alternatives for less-effective antibiotics, which are commonly used.

Keywords: Metallo-β-lactamases, Methanolic Extract, *P. aeruginosa*

1. Background

Pseudomonas aeruginosa is an important nosocomial pathogen. In recent decades, inappropriate use of antibiotics has led to drug resistance among bacteria, which is the grounds of high mortality rates throughout the world, particularly among people with suppressed immunity. The production of metallo-β-lactamases (MBLs) that confer resistance to all β-lactams except aztreonam is a mechanism of increasing clinical importance, mostly driven by the international spread of MBL producing organisms. Furthermore, the MBL-encoding genes that located on integrons can be disseminated easily from one bacterium to another. Many MBLs have been found in *P. aeruginosa*, including Australian imipenemase (AIM), (Verona integron-encoded metallo-β-lactamases (VIM)), Sao Paolo metallo (SPM), Seoul imipenemase (SIM), German imipenemase (GIM), Japan, Kyorin university hospital imipenemase (KHM), New-Delhi metallo-beta-lactamase-1 (NDM-1) and imipenemase (IMP). The genes of both IMP and VIM-type in clinical isolates of *P. aeruginosa* are usually encoded on mobile elements inserted into class 1 integrons. The integrons are located on transposons or plasmids, the distribution of which contributes to the wide spread of this resistance mechanism (1-4).

Zataria multiflora is a member of the Labiatae with a Woody, fibrous root, and its leaves are small, narrow, and elliptical, greenish-gray in colors. It grows in countries like Pakistan, Afghanistan and Iran. Traditionally, it has been utilized as treatment of sore throat, jaundice, chronic catharsis and asthma. *Z. multiflora* has been reported to have

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applied for medical properties including pain-relieving, immunostimulant, and antibacterial, anticandidal, antifungal and anti-inflammatory effects (4-6).

The genus *Ziziphus* belongs to the Rhamnaceae family. The members of this genus are drought tolerant and very resistant to heat. It is a small to medium-sized tree, with a spreading canopy. It has widely extended from South Africa northwards to Ethiopia and Arabia. The leaves of the plant are utilized in the treatment of diarrhea, wounds, abscesses, swelling and gonorrhea and they are also used in the treatment of liver diseases, asthma and fever (7, 8).

Chamomile is a member of the daisy family (Asteraceae or Compositae). It is a perennial herbaceous plant cultivated in western Europe and north Africa. Inward in traditional medicine, Chamomile is applied as an anti-inflammatory agent for stomach upsets. In women, the antispasmodic effects of Chamomile ease menstrual cramps, and lessen the possibility of premature labor also, Chamomile extract's stimulating effect on leukocytes (macrophages and b lymphocytes) and it is applied in skin irritations and eczema (9, 10).

Myrtle (*Myrtus communis* l.) is an evergreen shrub that belongs to the family of Mirtaceae that grows spontaneously. It is still extensively cultivated throughout the Mediterranean area. In classic medicine, myrtle has been shown to have anti-inflammatory effects. The anti-microbial activity of myrtle in *Escherichia coli*, *staphylococcus aureus*, *P. aeruginosa*, *Proteus Vulgaris*, *Proteus Mirabilis*, *Klebsiella aerogenes*, *salmonella typhi* and *Shigiella* has been determined (11, 12).

2. Objectives

The aim of this study was to define the antibiotic resistance patterns of *P. aeruginosa*, detect blaVIM and blaIMP MBL genes, and lastly evaluate the effects of the methanolic extracts of the leaves of *Z. Multiflora*, *Ziziphus*, Chamomile and *M. communis* on *P. aeruginosa* strains producing MBL (blaimp) isolated from the burn patients hospitalized in Shahid Motahari hospital, Tehran, Iran during 2011 - 2012.

3. Materials and Methods

3.1. Sampling Size

This is the random sampling, and the number of isolation was selected for this study according to the following Equation 1 (P = 0.5; d = 0.1):

$$n \geq \frac{Z^2_{1-\frac{\alpha}{2}} p(1-p)}{d^2} = \frac{3.8416 \times 0.5 \times 0.5}{0.12} = 96 \quad (1)$$

Data were analyzed using the chi-square, t-test, Fisher's exact test with SPSS software version 16 (SPSS Inc USA). P values of less than 0.05 were considered statistically significant.

3.2. Isolation and Clinical Identification

Ninety-six *P. aeruginosa* strains were isolated from 400 burn patients (men and women) referred to Shahid Motahari hospital (level I burn care center in Tehran, this is the main general hospital for the burning patients and also it is the main center for the burning research in Tehran, Iran since February 2012 till October 2013. Most of the samples were isolated during spring and summer due to the prevalence of burn patients in these seasons. Also, the higher rates of *P. aeruginosa* infection were observed during the hot months.

To prepare the samples, the wounds were washed with physiological serum. At first samples were transferred to culture media such as Cetrimid and MacConkey agar then incubated at 37°C for 24 hours. Then, we used biochemical tests including oxides, catalase, and growth ability at 42°C. *Pseudomonas aeruginosa* ATCC27853 was used as a control strain.

3.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), amikacin (AK, 30 μg), tobramycin (TOB, 10 μg), piperacillin/Tazobactam (PTZ, 100/10 μg), ciprofloxacin (CIP, 5 μg), cefepime (FEP, 30 μg), ceftriaxone (CRO, 30 μg), aztreonam (ATM, 30 μg), gentamicin (GEN,10 μg) and carbenicillin (Car,100 μg) (MastGroup, Merseyside, UK) was tested on the isolated *P. aeruginosa* samples, as well as the control ATCC27853 according to clinical laboratory standards institute (CLSI) guidelines.

3.4. Molecular Detection Methods

blaIMP and blaVIM genes were detected by polymerase chain reaction (PCR) method and DNA templates were prepared by Qiagene kit. The polymerase chain reaction amplification for blaIMP and blaVIM was performed with primers VIM-F (5′-GTTTGGTCGCATATCGCAAC-3′) and VIM-R (5′-AATGCGCAGCACCAGGATAG-3′) for blaVIM gene and primers IMP-F (5′GAAGGCGTTTATGTTCATAC-3′) and IMP-R (5′GTATGTTTCAAGAGTGATGC-3′).

3.4.1. Sequencing

The PCR purification and the sequencing were performed at the same company. (Bioneer Co., Korea) .The sequences were analyzed with Chromas 1.45 and MEGA- 4 softwares and BLAST at PubMed NCBI.

3.5. Plant Materials

The leaves of *Z. multiflora*, *Ziziphus*, Chamomile and *M. communis* plants were collected from the Fars Province in Iran, during 2012. The leaves of the plants were dried at 25°C and then powdered using a mechanical grinder. Ten gram of each powder sample was soaked in 100 mL of methanol (96%, v/v) from (Merck, Germany). The mixture

is putted for 48 hours in a dry place. The solution was filtered at first by Whatman No. 1 filter paper to clarify and then through a 0.45 μm membrane filter. Then, it was filtered through a filter paper slowly. Extracts obtained separately were poured into Petri dishes and dried in laboratory space.

4. Results

From a total of 400 patients, 96 *P. aeruginosa* strains were isolated, that 83 were resistant to imipenem and ceftazidime. The combination disk diffusion test showed that among the 83 imipenem which are non-susceptible *P. aeruginosa* strains, 48 (57.9%) were MBL producers. All MBL-producing *P. aeruginosa* strains were resistant to meropenem, imipenem, ceftazidime, amikacin, tobramycin, ciprofloxacin, aztreonam, piperacillin/tazobactam, ceftriaxone, cefepime and carbenicillin; while 49% of isolates were resistant to gentamicin, indicating that 100% of isolates were multi-drug resistant (MDR) (resistance to more than three antibiotics from different classes was defined as MDR). The minimum inhibitory concentration (MIC) of different antibiotics for IMP-producing *P. aeruginosa* strains is shown in Table 1. Using the PCR method, 6 isolates were positive for bla (IMP) gene, while bla (VIM) gene was not detected. Sequencing of PCR products showed a conserved region of the restriction sequence blaIMP-1 gene that was confirmed by the BLAST. Forty-eight patients (57.9%) were infected with MBL-producing Pseudomonas strains, of whom 4 (8.3%) died. The antibacterial potency of *Z. multiflora*, *Ziziphus*, Chamomile and *M. communis* extracts against six IMP-producing *P. aeruginosa* strains were evaluated by the microdilution method as described by CLSI. The results of MICs and MBCs (mg/mL) of Chamomile and *M. communis* against IMP-producing *P. aeruginosa* strains have presented in Table 2, while *Z. multiflora*, *Ziziphus* did not show any significant effect on these 6 isolates.

Table 1. Distribution of Minimum Inhibitory Concentrations of Antibiotics for IMP-Producing *Pseudomonas aeruginosa* Strains

Antibiotics	Minimum Inhibitory Concentration, μg/mL					
	P. a FSH2IMP	***P. a FSH22IMP***	***P. a FSH28IMP***	***P. a FSH40IMP***	***P. a FSH42IMP***	***P. a FSH47IMP***
Imipenem	128	128	128	128	128	128
Meropenem	64	64	64	64	64	64
Cefepime	128	128	128	128	128	128
Ceftazidime	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256
Cefotaxime	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256
Ampicillin	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256
Piperacillin/Tazobactam	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256
Ceftriaxone	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256

Table 2. Frequency of Minimum Inhibitory Concentrations of *Myrtus communis*, *Zataria multiflora*, *Ziziphus* and Chamomile Extracts for IMP-Producing *Pseudomonas aeruginosa* Strains

Strain	*Myrtus communis*		*Zataria multiflora*		Chamomile		*Ziziphus*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
P.a FSH2IMP	6.25	12.5	6.25	12.5	12.5	15	12.5	25
P.a FSH22IMP	6.25	12.5	6.25	12.5	12.5	25	12.5	25
P.a FSH28IMP	6.25	12.5	6.25	6.25	12.5	25	12.5	25
P.a FSH40IMP	6.25	12.5	6.25	12.5	12.5	25	12.5	25
P.a FSH42IMP	6. 25	NA	6.25	6.25	12.5	25	12.5	25
P.a FSH47IMP	6.25	NA	6.25	6.25	12.5	25	12.5	25
***P.aeruginosa* ATCC27853**	6.25	12.5	1.56	NA	12.5	25	12.5	25

Abbreviation: NA, not available.

5. Discussion

Pseudomonas aeruginosa is an opportunistic pathogen and one of the most important causes of infection in burn patients that followed by *Staphylococcus aureus* and *Acinetobacter baumannii* (13). *Pseudomonas aeruginosa* acquired antibiotic resistance; so, we need new methods of treatment to decrease the probability of drug resistance. MBL producer *P aeruginosa* in burn patients is the main reasons for increasing mortality and morbidity rates. In the two last decades, *P aeruginosa* was the most dominant bacteria in burn patients in Tehran, Iran (14). All of the *P. aeruginosa* strains in our study have resistance against almost all antibacterial agents. The MBL-producing *P aeruginosa* strains were resistant to amikacin, ciprofloxacin, ceftazidime, tobramycin, imipenem, meropenem, ceftriaxone, carbenicillin, piperacillin/tazobactam and cefepime. Also, 49% of the isolates were resistant to gentamycin. *P aeruginosa* have some mechanisms that can cause drug resistance such as enzyme mechanism and Efflux pump iron which have the ability to develop resistance to antibacterial agents (15). We have a high rate of MBLs in our study in comparison to some studies in other parts of the world, the study in Spain showed that just 6.9% of isolates were MBL producer (16), in India MBL producer were 33% (17), but the rate of MBL producer was lower in our study, maybe because of treatment policy such as antibiotics that prescribed and hospitalization condition. The most reported, as well as in Iran indicated that the prevalence of VIM beta-lactamase is more than IMP (18, 19) but in our study IMP was the most dominant MBL that is in concordance with other studies. In our study, 6 isolates were positive for bla (IMP) gene. Some other genes probably can cause resistance such as GIM, KHM, SIM, AIM, SPM, NDM and FIM (20-22). The mortality rate of infection due to MBL-producer *P aeruginosa* in Spain was 27% (23), in Brazil was 82.6% (24) and in our study was 8.3%. Also, VIM-2 can cause drug resistance; the existence of this gene in *P. aeruginosa* was reported in France for the first time (25). In our study, the antibacterial effect of, *Z. multiflora* plants and *M. communis*, Chamomile, *Ziziphus* leaves were tested against MBL-producer *P. aeruginosa*. We conclude that *Myrtus communis* extracts had a beneficial antibacterial effect against regular and IMP-producing *P. aeruginosa* strains. Kang et al. used ethanolic extracts of *M. communis* and inhibitory growth of *P. aeruginosa* was observed (26). Akin et al. concluded that *M. communis* essential oil was not a good inhibitor for *P. aeruginosa*; however, we found *M. communis* as a good inhibitor in our study (27). Owlia et al. has shown that *M. communis* had an antibacterial effect on this isolates, but Chamomilla essential oils were effortless on *Pseudomonas aeruginosa* that is in contrast to our study (28). Hashemi et al. reported that the inhibitory effect of *Z. multiflora* on *P. aeruginosa* isolated from burn patients are more than *Peganum harmala* and *M. communis* (29). In the same study to our research Al-Saimary et al. in 2001 in Iraq found that the aqueous extracts of *M. communis* and Eucalyptus leaves had a good effect on *P aeruginosa* that isolated from burned patients (30). Bokaeian et al. in 2014 suggested that *M. communis* leaves are powerful bactericidal and effective against *P. aeruginosa* and Klebsiella pneumonia (31). Carvalho et al. reported that ethanolic extract of Chamomile had a beneficial antibacterial effect against *P. aeruginosa* and no effect against *S. aureus*, *E. coli*, *Salmonella enterica* subsp. *enterica* sorovar Typhimurium (32).

5.1. Conclusions

As the IMP producing *P. aeruginosa* is increasing in burn patients, detection of them is absolutely important to identify *P. aeruginosa* drug resistance, which can show and improve developing methods of drug therapy as alternative models for the physicians to avoid synthetic resistance drugs for patient treatment. The methanolic extract of *Z. multiflora* and *M. communis* had more beneficial effect on clinical IMP-producing *P. aeruginosa* strains compared to the routine approach in this bacteria treatment. Therefore, these herbal extracts can be the best alternatives for the traditionally less-effective antibiotics, which are normally used till now.

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Footnotes

Authors' Contribution: All of the authors cooperating in all part containing taking sample, isolation of the sample, the practical working of preparing extract, and also writing the article.

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