The antibacterial activity of Iranian plants extracts against metallo beta-lactamase producing *Pseudomonas aeruginosa* strains

Mohsen Heidary[1], Ali Hashemi[1], Hossein Goudarzi[1], Saeed Khoshnood[1], Mahdaneh Roshani[1], Hadi Azimi[2], Mehdi Goudarzi[*,1]

[1]Depatrment of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of English Language Teaching, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding Author: email address: gudarzim@yahoo.com (M. Goudarzi)

ABSTRACT

Metallo β-lactamases (MBLs) producing *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates are becoming an escalating global threat. Among the antibiotics used to treat infections associated with *P. aeruginosa*, resistance to carbapenem is a serious therapeutic challenge. The aim of the present study was to detect MBL-producing *P. aeruginosa* and to evaluate the extracts of *Urtica. dioica*, *Carum. copticum*, and *Zataria multiflora* on these clinical pathogens. The study was performed on hospitalized burn patients during 2014. Antibiotic susceptibility testing was assessed by broth micro dilution and disc diffusion methods. The MBLs were detected using combination disk diffusion test (CDDT) phenotypically. Then, PCR and sequencing methods were carried out to detect the MBL encoding genes. Among 83 imipenem resistant *P. aeruginosa* strains, 48 (57.9%) isolates were MBL-producing *P. aeruginosa*. PCR and sequencing methods confirmed that these strains were bla_{IMP-1} positive genes, whereas none were positive for bla_{VIM} genes. Hospitalized burn patients with MBL-producing *P.aeruginosa* infection had 4/48 (8.3%) mortality rate. It was demonstrated that *C. copticum*, *U. dioica*, and *Z. multiflora* extracts had significant antibacterial effects on regular and IMP-producing *P. aeruginosa* strains. The prevalence of MBL-producing *P. aeruginosa* isolates in burn patients is generally very high. All MBL-producing strains encode the bla_{IMP-1} gene. Therefore, detection of MBL-producing strains has major importance in identifying drug resistance patterns in *P. aeruginosa* and in controlling of infections. In the current study, the extracts from *C. copticum*, *U. dioica*, and *Z. multiflora* had high antibacterial effects against β-lactamase producing *P. aeruginosa* isolates.

Keywords: *Zataria multiflora*; *Urtica. dioica*; *Carum. copticum*; *Pseudomonas aeruginosa*; metallo β-lactamase

INTRODUCTION

P. aeruginosa is an opportunistic bacterium that causes different conditions such as septicemia, pneumonia, urinary tract infection (UTI), endocarditis, and skin-eye-ear infections [1]. This bacterium is one of the major causes of hospitalized infections and the main cause of death in patients with cystic fibrosis, severe burns, and AIDS [2]. *P. aeruginosa* is one global threat of hospitalized infections in the entire world [3]. It is estimated that about 63,000 people in the U.S. and 25,000 in the E.U. die each year because of the infectious diseases caused by multi-drug resistant (MDR) bacteria [4]. MBLs are the genes causing resistance in *P. aeruginosa*. The MBL-encoding genes, bla_{IMP} and bla_{VIM}, which are often located on integrons, can easily spread among bacteria [5]. Both genes of *imp* and *vim*-type in *P. aeruginosa* clinical strains are often located on class 1 integrons [6]. The integrons are motionless per se, yet as they are located on transposons or plasmids, they would be mobile and thus can spread the resistance [7].

This is in line with the existence of herbal medicine that can possibly be helpful in treatment of infections produced by MDR isolates. Using herbs to treat diseases has long been common among mankind. Over the ages, the beneficial effects of various plants and herbs for treatment have been realized. Modern life has led to the reduction in consumption of medicinal plants. They are now replaced by synthetic drugs. Taking these drugs has unfortunately increased

the resistance of microorganisms and reduced the effects of medications [8].

Treatment based on medicinal plants has commonly been cheaper, easier, and more accessible with fewer side effects in comparison with chemical compounds. On the other hand, people seem to be more inclined to use herbal remedies compared to chemical drugs [9].

C. copticum grows in east India, Egypt, and Iran, with brilliant flowers and brownish seeds, having an odour like thymol. Its essential oil includes β-pinene, paracymene, terpinene, α-pinene, and other components such as thymol and carvacrol. The seeds are used as diuretic, analgesic, anti-asthmatic, anti-vomiting, and anti-dyspnea agents. This plant has a great effect on skin-related infections and urinary tract disorders [10-12]. *U. dioica* belongs to Urticacease family, an herb that has long been noted. In Europe, this herb is used to reduce inflammation and to treat rheumatoid arthritis [13, 14]. *U. dioica* in Iranian traditional medicine is used in the treatment of diabetes. This is an anti-inflammatory, blood sugar and blood pressure lowering, diuretic, analgesic, local anesthetic, anti-prostate inflammation plant. Recently, *U. dioica* has been used for the treatment of infectious diseases and reduction of arthritis symptoms and joint inflammation. Other medicinal uses of this herb are in the treatment of bladder and urinary tract infections, prostate enlargement, seasonal allergies, and acne [15]. *U. dioica* grows in the northern west and central parts of Iran. Its combinations include hydrophilic compounds such as flavonoids, lecithin, polysaccharides, acetic acid, and butyric acid. *Z. multiflora*, a member of the Labiatae family, is grown in Afghanistan, Pakistan, and Iran. Conventionally, it has been used to treat chronic catharsis, premenstrual pain, edema, sore throat, jaundice, and asthma. This herb is used as diuretic, antiseptic, flavoring, carminative, and as an antispasmodic agent. *Z. multiflora* has been described to have applicable medical properties including pain-relieving, immunostimulant, antibacterial, anticandidal, antifungal, antioxidant, antinociceptive, and anti-inflammatory effects [16-18]. The aim of the present study is to detect MBL-producing *P. aeruginosa* by PCR and to evaluate the antibacterial effects of acetone and methanol extracts of *U. dioica*, *C. copticum*, and *Z. multiflora*, Iranian medicinal herbs, on *P. aeruginosa* clinical isolates.

MATERIALS AND METHODS

Collection and identification of bacteria

The current study was carried out on 448 hospitalized burn patients who had referred to Shahid Motahari Hospital in Tehran, Iran. During the year 2011, 100 strains of *P. aeruginosa* were collected from these patients. Wounds were washed by physiological serum and then sampling was done using a sterile swab. The swabs were placed in Stuart transport medium and transferred to the laboratory of Microbiology Dept., Shahid Beheshti University of Medical Sciences, Tehran-Iran. The samples were cultured on Cetrimide and MacConkey agar and incubated at 37ºC for 24 hours. Colonies were evaluated for the presence of *P. aeruginosa* by the differential and diagnostic tests. Pigmented and odoriferous colonies were analyzed using biochemical tests, including oxidase, catalase, sugar fermentation, and growth ability at 42°C tests. The strains were placed at -20ºC in brain heart broth, together with 20% glycerol. *P.aeroginosa* ATCC27853 was selected as the reference strain.

Disk diffusion method

According to the guidelines of the CLSI [19], antibacterial susceptibility to selected antibiotics (Mast Group, Merseyside, UK) was tested on the *P. aeruginosa* isolates as well as the control ATCC27853 strain by disk diffusion method on Mueller Hinton agar (Merck, Germany) [19]. MBL detection was performed through Combination Disk Diffusion Test (CDDT). In this method, Imipenem and Imipenem plus EDTA discs were used to detect MBL producing *P. aeruginosa*. MDR is defined as resistance to three or more than three antibiotics from various classes.

Antibiotics MIC determination

Sensitivity of 100 *P. aeruginosa* strains was performed based on the minimum inhibitory concentration (MIC) by broth micro dilution method according to CLSI guidelines [19]. Certain doses of imipenem, meropenem, aztreonam, cefotaxime, and ceftazidime were added to the Muller Hinton agar (GLAXO England Co. and Himedia Co.).

A total of 10 ml of bacterial suspension was inoculated on Mueller-Hinton agar medium containing different dilutions of drugs using inoculators hand apparatus (Co MAST). Plates were examined after incubation at 37°C for 18 hours.

PCR and Sequencing

Polymerase chain reaction (PCR) method was performed for detection of bla_{VIM} and bla_{IMP} genes in *P. aeruginosa* strains by using of temperature and the genes specific primers. Half µl of extracted DNA plus 0.5 µl from each primer were uses. PCR master mix was then added to each tube (South Korea Bioneer Company) to a final volume of 20 µl.

The PCR amplification for bla_{IMP} and bla_{VIM} was done with Primers VIM-F (5′-GTTTGGTCGCATATCGCAAC-3′) and VIM-R (AATGCGCAGCACCAGGATAG-3′) for bla_{VIM} gene and primers IMP-F (5′-GAAGGCGTTTATGTTCATAC-3′) and IMP-R (5′-GTATGTTTCAAGAGTGATGC-3′) for bla_{IMP} gene under PCR conditions, as previously reported (20). The PCR purification kit (Bioneer Co., Korea) was used to purify PCR products and sequencing was performed by the Bioneer company (Korea). The nucleotide sequences were analyzed by Chromas 1.45 and MEGA-4 as well as BLAST in NCBI.

Collection and Identification of Plants

During the year 2012, *U. dioica*, and *Z. multiflora* leaves and *C. copyicum* seeds were collected from Fars and Guilan provinces in Iran. A voucher specimen was prepared and deposited at Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Extraction Method

U. dioica and *Z. multiflora* leaves and *C. copyicum* seeds were dried at room temperature and powdered. Twenty grams of each powdered herb were solved in methanol and acetone solvents, and then placed in the dark for a period of 48 hours without heating. Next, it was purified and placed at room temperature to dry and was left at -20°C until performing the following tests.

Extracts MIC Determination

The MIC of the extracts was evaluated according to the method reported by CLSI, 2012. *C. copyicum*, *U. dioica*, and *Z. multiflora* extracts were dissolved in DMSO 10% and then a certain amount of prepared solution was added to Mueller Hinton agar medium. Concentrations were mixed thoroughly in the media containing extracts. Ten µl of suspension (equivalent to 0.5 McFarland) was added to plates containing extracts and incubated at 37ºC for 24 hours. Finally, the effect of extracts was studied against *P.aeruginosa* strains isolated from burn patients.

Statistical Analysis

The effect of *Z. multiflora*, *U. dioica*, and *C. copticum* extracts on *P. aeruginosa* was analyzed via one-way ANOVA using MINITAB (version 13). A p value <0.05 was considered significant.

RESULTS

Among 100 *P. aeruginosa* strains, 83 were imipenem and ceftazidime resistant. The CDDT demonstrated that from among 83 Imipenem resistant *P. aeruginosa* isolates, 48 (57.9%) were MBL producers. All MBL-producing *P. aeruginosa* isolates were resistant to ceftazidime, ceftriaxone, cefepime, meropenem, imipenem, amikacin, tobramycin, ciprofloxacin, aztreonam, piperacillin/tazobactam, and carbenicillin. The results showed that 100% of strains were MDR. The MIC of selected antibiotics for IMP-producing *P. aeruginosa* strains are shown in Table 1. The results of PCR show that 6 strains were positive for bla_{IMP} gene, while bla_{VIM} gene was not detected.

Table1. Distribution of antibiotics MICs for IMP-producing *P.aeroginosa* strains.

	Antibiotics	**MIC[a](µg/ml)**					
		P.a[b] FSH[c]2IMP[d]	**P.a FSH22IMP**	**P.a FSH28IMP**	**P.a FSH40IMP**	**P.a FSH42IMP**	**P.a FSH47IMP**
carbapenems	**imipenem**	128	128	128	128	128	128
	meropenem	64	32	64	64	64	64
cephalosporins	**cefepim**	128	128	128	128	128	128
	ceftazidime	≥256	≥256	≥256	≥256	≥256	≥256
	cefotaxime	≥256	≥256	≥256	≥256	≥256	≥256
	ceftriaxon	≥256	≥256	≥256	≥256	≥256	≥256
penicillins	**ampicillin**	≥256	≥256	≥256	≥256	≥256	≥256
	piperacillin-tazobactam	256	256	256	256	256	256

[a]minimum inhibitory concentration; [b]*Pseudomonas aeuroginos;* [s]pecial code for *Pseudomonas aeroginosa* imipenem resistant; [d] Imipenem

Table 2. Frequency of minimum inhibitory concentration (MIC mg/ml) of *C. copticum* , *U. dioica*, and *Z. multiflora* extracts for IMP-producing *P.aeruginosa* strains

	C. copyicum		***U. dioica***		***Z. multiflora***	
Strain	**Methanol (mg/ml)**	**Acetone (mg/ml)**	**Methanol (mg/ml)**	**Acetone (mg/ml)**	**Methanol (mg/ml)**	**Acetone (mg/ml)**
P.a FSH2IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.a FSH22IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.a FSH28IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.a FSH40IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.a FSH42IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.a FSH47IMP	6.25	12.5	2.5	1.25	6.25	6.25
P.aeuginosa ATCC27853	1.56	6.25	1.56	1.56	1.56	1.56

Forty eight (57.9%) patients were found to be infected with MBL-producing *Pseudomonas* isolates among which four (8.3%) died in the course of the study. Antibacterial activity of *C. copticum*, *U. dioica*, and *Z. multiflora* extracts against six IMP-producing *P. aeruginosa* isolates were determined by the micro dilution method as described by CLSI [20]. The results of MICs (mg/ml) for *C. copticum*, *U. dioica*, and *Z. multiflora* against IMP-producing *P. aeruginosa* strains are presented in Table 2.

DISCUSSION

Although the use of antibiotics for treatment of clinical infections seems to be safe and effective, it has created several problems despite their benefits. With the expanding use of synthetic antibiotics, the number and type of bacterial resistance to drugs have been raised insofar as need for new antibacterial agents are indisputable. In addition to different progressed methods for obtaining new synthetic drugs, the use of herbal medicines for this approach can be considered valuable. Grappling with infectious diseases and emergence of resistant bacteria and the side effects of antibiotics explains the need for new antibacterial agents with natural origins. [21]. MBL producing isolates of *P. aeruginosa* are a main threat in hospitals and are a cause of high morbidity and mortality [22]. During the past two decades, *P. aeruginosa* was the most common bacteria among burn patients in Iran. *P. aeruginosa* strains studied in the present study appeared to be resistant to almost all antibacterial drugs. All MBL-producing *P .aeruginosa* were resistant to meropenem, imipenem, ceftazidime, ceftriaxone, cefepime, amikacin, tobramycin, ciprofloxacin, aztreonam, piperacillin/tazobactam, and carbenicillin. The prevalence of drug resistance in burn wards has been reported from other parts of the world, too [7, 23]. Several studies have demonstrated that the prevalence of MBL-encoding genes in *P. aeruginosa* strains can be different from one country to another [24]. Compared with other study, a higher prevalence of MBLs was found in our study. It can be attributable to various hospitalization time and treatment protocols [25-28]. The VIM beta-lactamase in Iran, as is the case in the whole world, is the most type of MBL [29-31]. Other investigations in Iran reported VIM-type positive *P. aeruginosa* strains from Tehran and Ahwaz 11.43% and 19.51% respectively. However, in the current study, IMP-1 producers of *P. aeruginosa* isolates were the most prevalent type among the strains studied, which is in contrast to findings reported in other studies [32-34]. In the present survey, 42% of strains were identified bla_{VIM} and bla_{IMP} negative; this resistance may be generated by other MBL encoding genes. Although mortality rate due to infection with MBL-producing *Pseudomonas* isolates was reported 82.6 % in Brazil, but in the current study was 4 (8.3%) [35].These enzymes-encoding genes located on plasmids can easily spread resistance and, in outbreaks, can lead to failure in treatment and consequently result in mortality rates up to 75% [36].There is few effective drugs against the MBL-producing *P. aeruginosa*. Consequently, it is safe to claim that there is a need to correct treatment protocols for resistant genes. Also, the antimicrobial effects of *C. copticum*, *U. dioica*, and *Z. multiflora* have been shown in many other studies [16, 17]. Nevertheless, antibacterial activity of these plants against IMP-producing *P. aeruginosa* isolates has not been reported yet. The present study confirms the idea that *C. copticum*, *U. dioica*, and *Z. multiflora* extracts can be useful as antibacterial agents on IMP-producing *P. aeruginosa* isolates. The acetonic and methanolic extracts of *Z. multiflora*, *U. dioica*, and *C. copticum* have hopeful MIC values on all IMP-

producing *P. aeruginosa* strains. The results of the study by Proesto et al. showed that the methanolic extract of *U. dioica* on *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* has strong antibacterial activity [37]. In another investigation, Akrami et al. reported that *P. aeruginosa* was inhibited from 4% to 6% (w/w) of Zataria essential oil in the active coating [38]. Moreover, Azizkhani et al. have shown that The MIC and MBC of *Z. multiflora* Boiss against Staphylococcus were 0.03 and 0.04%, respectively [39]. In addition, the effective activity of ethanol extract of *U. dioica* against *P .aerogenusa*, *E .coli*, and *S .aureus* and *Salmonella spp* were investigated by Gorzalczany et al [37].

"The authors declare no conflict of interest"

CONCLUSION

The prevalence of MBL-producing *P. aeruginosa* in hospitalized burn patients is high. Thus, evaluation of these strains has a major importance in the detection of antibiotic resistance patterns controlling *P. aeruginosa* infections. It was observed that the acetonic and methanolic extracts of Zataria multiflora, *U. dioica* and, *C. copticum* had a significant effect on clinical MBL-producing *P. aeruginosa* isolates.

A natural drug with higher efficiency can be a good substitute for the chemical drugs, with all their side effects, commonly used in vivo investigations. Further studies should aim at introducing proper herbs that will help to produce such natural drugs.

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