

Original Article

Antimicrobial Effect of Methanolic and Acetonic of *Zataria Multiflora*, *Capsicum Annum L.* and *Piper Nigrum L.* Extracts on *Pseudomonas aeruginosa* Strains Isolated from Patients Hospitalized in the Burn Ward of Shahid Motahari Hospital in Tehran

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Abstract

Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the main causes of nosocomial infection. Burn patients are at high risk of acquiring this bacterium due to skin damage and their immune deficiency, and mortality rate in these infected patients is high (40-50%). Therefore, due to antibiotic resistance of MBL containing strains in this bacterium, the aim of this study is to investigate the effect of methanol and acetone of *Zataria multiflora*, *Capsicum annum L.* and *Piper nigrum L.* on strains containing MBL in this bacterium.

Materials and Methods: This lab study was conducted on samples from burn patients, which were gathered between 2015 and 2016. In this study first, disc diffusion and MIC were done based on the CLSI protocol; and using a combined disk, we detected metallo-beta-lactamase. Next, the *bla*_(IMP) and *bla*_(VIM) genes were identified by the PCR method. In order to investigate the effect of three plants extract on bacteria, the bacteria was affected by triple extracts using MIC and disk diffusion.

Results: According to the results, all three plants had an acceptable effect on *Pseudomonas aeruginosa* strains containing metallo-beta-lactamase, and to be more precise, the acetone type of extract of *Capsicum Annum L.* at a concentration of 1.5 mg / ml had the best effect in treating of these bacteria.

Conclusion: The results of this study indicate the presence of several mechanisms of resistance to beta-lactam antibiotics among *Pseudomonas aeruginosa* strains collected from burn patients. The emergence of these types of XDRs has led to health problems, especially in burn patients. According to the results, the methanolic and acetonic extract of all three plants have been shown to be effective in inhibiting the growth of MBL-containing *Pseudomonas aeruginosa*.

Keywords: metallo-beta-lactamase, *Pseudomonas aeruginosa*, *Zataria multiflora*, *Capsicum annum L.*, *Piper nigrum L.*

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Introduction

Hospital infections are one of the major problems in health care centers. Hospital infection is an infectious disease that the patient does not develop at the time of admission, but it occurs 48 to 72 hours after the patient admits to the hospital, three days after discharge or thirty days after the surgery. Despite much care, hospital infections have been significantly associated with complications and mortality, and impose a high cost on the patient. Currently, in developed countries, the rate of these infections is about 10% admitted and in developing countries are estimated around 25%¹⁻³. The main microorganisms responsible for the hospital infection include *Pseudomonas*, *Candida*, *Escherichia coli*, *Enterococcus*, *Klebsiella*, *Enterobacter* and *Staphylococcus*⁴.

The burn and its complications are one of the most common causes of death in the world; burns are the fifth cause of death due to injuries. Hospital infections are one of the most commonly encountered complications of hospitalization and cause mortality in those patients, which are associated with a greater incidence of burns due to burn injuries caused by burn injuries. Today, it proves that 75% of deaths due to burn are associated with an infection, which occurs after septic shock and severe bacteremia.

Pseudomonas aeruginosa is the most common bacteria in infectious diseases. The bacterium is found in all hospital environments in wet containers such as foods, flowers, bath tubs, toilets, salts, and respiratory and dialysis treatments, and even in disinfectants. *Pseudomonas* extensive environmental dissemination is probably due to simple growth needs and nutritional adaptability. They can use many organic compounds as sources of carbon and nitrogen, even some species can grow in distilled water using little materials⁵. These bacteria differ in terms of dietary requirements, and almost all of them grow in the presence of ammonium salts and a simple organic source. These bacteria are highly aerobic and use the oxygen molecule as the final electron receptor. Some species of *Pseudomonas* can grow in the anaerobic environment in the presence of nitrate or arginine⁶⁻⁷.

Pseudomonas aeruginosa is more associated with human pathogenicity than other species of *Pseudomonas*. The bacterium in humans naturally exists and is saprophytic, which sometimes causes the disease in people with an impairment of immunity⁸. Epidemiologic studies have shown that this bacterium is responsible for 10% of hospital infections, 11% of blood infections and 4% of hospitalized epidemics⁹. This bacterium causes infections such as pneumonia, urinary tract infections, bacteremia, endocarditis, and skin, ear, and eye infections in hospitalized patients¹⁰. In addition, this bacterium causes inflammation, stimulates the immune system and produces skin ulcers in patients with burns¹¹. In burned patients who infected by this bacterium, it will be very difficult treat them, and mortality rates in these patients will be higher than 40%¹².

Pseudomonas aeruginosa has inherent resistancy to many antibiotics and can become more resistant over the time. The mutation of purine proteins is the main mechanism of resistance. *Pseudomonas* can also inhibit many beta-lactam antibiotics, such as Penicillin, Cephalosporin, Carbapenem, by producing various beta-lactamases¹³. The mechanism of antibiotic resistance of this bacterium to antibiotics is to reduce the permeability of the extracellular membrane, leakage pumps, changes in the location of the antimicrobials and the change in the antibiotic structure before reaching the target¹⁴.

Although antibiotics are the first-line treatment for hospital infections caused by this bacterium, long-term use of synthetic antibiotics may have many side effects. Therefore, an alternative treatment based on natural products is needed.

Zataria multiflora or broad-leaved thyme has a set of green leafy leaves, legs and shoots, and white oval-shaped white flowers. The flowered branches of this plant are from the labiatae family¹⁵⁻¹⁶. It is also swollen to relieve stomach troubles, bone pain, and eating cold cuts. In addition, in some topical products, it is used as anticancer, antimicrobial, rubs and stimulants, and solutions are used to reduce swelling of the throat and mouth¹⁷.

Capsicum annum L. are spiced and reddish fruits with a bitter smell and taste. This plant is from Solanaceae¹⁸. Herbs are often herbaceous plants, one-year-old, or rugged, or rarely raised, or have entirely

woody or tree-like organs. *Capsicum annum L.* is herbaceous plant up to 60 cm high, growing in tropical areas, but it cultivated in most parts of the world, including Iran. Its leaves are sharp, elliptical, and its flowers are yellowish and fleshy, spiced and greenish, yellow and reddish-green, and rich in flat and white seeds¹⁸. *Capsicum annum L.* is used to treat indigestion due to muscle weakness. Due to the presence of the active ingredient of capsaicin, which has local stimulant properties, it can be used to relieve rheumatic pain, back pain and pain from peripheral neuropathy. Capsaicin, by binding to a specific protein receptor, which is usually activated at a temperature above 37°C, causes the protein to be stimulated at a lower temperature and excite a thermal stimulation signal to the brain. This is the reason why you feel warm when you eat peppers. Capsaicin causes the planned death of prostate and lung cancer cells. According to studies, capsaicin works for reduce blood glucose levels after eating food¹⁸.

Piper nigrum L. is a small, spherical, black colored fruit or powder from these fruits that has a fragrant flavor and aroma. This pepper, a growing herb and with its scroll roots, finds some kind of coexistence or parasitic life with surrounding plants¹⁸. This pepper is 1.5% organic, with a pleasant smell but with a warm and burning taste. The bulk of this essential oil is also produced by Flandren and Cadine Ne. In pepper, in addition to the essential oil, there is also a soluble resin in ether and alcohol, with a spicy flavor, an alkaloid called Pipe Rine. This pepper has a stimulating and sneezing effect and its use for people with gastrointestinal diseases as well as hemorrhoids, soda and tuberculosis should be banned¹⁹.

This study was conducted on hospitalized burn patients in Shahid Motahari Hospital, Tehran, Iran, from 2015 to 2016. Three aims have been considered in this study: firstly, to determine the antibiotic resistance patterns of *P. aeruginosa*, secondly to detect *bla_{VIM}* and *bla_{IMP}* metallo-beta-lactamase genes, and thirdly to evaluate the effects of *Z. multiflora*, *Capsicum annum L.* and *Piper nigrum L.* methanolic and acetonic extracts on *P. aeruginosa* strains producing metallo-β-lactamase (*bla_{IMP}*) isolated from the studied patients.

Methods

Initially, the standard strain of Lyophilized *Pseudomonas aeruginosa* was prepared from the Iranian Scientific and Industrial Research Organization and the microbial culture steps were performed on the Muller Hinton agar medium.

Sampling and performing bacterial identification tests: After examining the burn patients referred to Shahid Motahari Hospital between January 2015 and January 2016, 100 isolates of *P. aeruginosa* were collected using sterile swabs. Samples were cultured in Ceramide and Mac-Conkey agar media and incubated at 37 ° C for 24 hours.

Antimicrobial susceptibility testing: In this study, antimicrobial susceptibility to antibiotics included to Imipenem (IPM, 10μg), Meropenem (MEM, 10μg), Ceftazidime (CAZ, 30μg), Amikacin (AN, 30μg), Doripenem (DOR, 30μg), Ticarcillin (TC, 10μg), piperacillin/Tazobactam (PTZ, 100/10μg), Ciprofloxacin (CIP, 5μg), Cefepime (CPM, 30μg), Piperacillin (RRL, 30μg), Aztreonam (ATM, 30μg), Gentamicin (GEN, 10μg) and Colistin (CO, 100μg) (Mast Group, Merseyside, UK) was tested on the isolated *P. aeruginosa* samples, as well as the control ATCC27853 strain by the Kirby-Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany) based on Clinical Laboratory Standards Institute (CLSI) guidelines²⁰.

Minimum Inhibitory Concentration (MIC) of Antibiotics: Minimum inhibitory concentration (MIC) of different antibiotics was determined according to the guidelines of the CLSI by broth microdilution method²⁰. Distinct doses of Imipenem, Meropenem, ciprofloxacin and Ceftazidime (GLAXO England Co. and Himedia Co.) were added to the culture medium (Muller-Hinton broth (Merck, Germany) Based on CLSI guidelines²⁰.

Phenotypic detection of MBL: In this study, Imipenem and Imipenem+EDTA discs were used to identify *Pseudomonas aeruginosa* strains producing MBL. MBL detection method is via Combination Disk Diffusion Test (CDDT).

Detection of *bla_{IMP}* and *bla_{VIM}* Genes by PCR Method: DNA templates were prepared using boiling method. The polymerase chain reaction (PCR) amplification for *bla_{IMP}* and *bla_{VIM}* were performed

with primers VIM-F (5'-GATGGTGTGGTTCGCATA-3') and VIM-R (5'-CGAATGCGCAGCACCAG-3') for *bla_{VIM}* gene and primers IMP-F (5'-GGAATAGAGTGGCTTAAAYTCTC-3') and IMP-R (5'-GGTTTAAAYAAAACAACCACC-3') for *bla_{IMP}* gene under PCR conditions as reported previously.

Sequencing: PCR purification kit (Bioneer Co., Korea) was used to purify the PCR product; and the Bioneer Company (Korea) performed forward strand sequencing.

Plant material

Source, Collection and Identification: The *Zataria multiflora* from herbal shop in Shiraz, Fars province, and *Capsicum annum L.* and *Piper nigrum L.* from herbal shop in southern Khorasan Province during 2016 were prepared. A botanist at Shahid Beheshti University in Tehran identified the plants.

Extraction Method: In this study, maceration method was used for extraction. Maceration is an old method that is performed by water or various solvents. In this method, the plant is poured or crushed into a large container and the amount of solvent is added to the extent that the solvent is covered with the whole powder and some amount is placed on the powder. This procedure is known in Pharmacopeia as the Maceration process²¹. This way, three plants were dried in the shade at room temperature (25C). Then, using the mill, powdered, 10 gr of dried plants were added and in 100 ml of the tested solvents (96% methanol, 99% acetone and 99.4% chloroform) added to the solvent, the total powder cover and put some on the powder, soaked. After 48 or 72 hours (per plant type), the solution was filtered with Whatman NO.1 filter paper. Then the solutions were filtered by a membrane filter. The micrometer was sterilized. Subsequently, these solutions evaporated in a vaporizer under vacuum conditions and reduced pressure. The dried extract was then weighed based on the desired concentrations and dissolved in DMSO 2% solvent and obtained the desired concentrations.

Determination of MIC and MBC values by broth Microdilution assay: The minimum inhibitory concentration (MIC) of the extracts was determined according to the method explained by CLSI, 2012²⁰. *Zataria multiflora*, *Capsicum annum L.* *Piper nigrum L.* extracts were diluted with DMSO 2% to yield concentrations ranging from 25 to 0.09 mg/ml. Cation-adjusted Muller Hinton broth was used as broth medium. After shaking, 0.1 ml of each extract was added to one well of 96-well microplates. Microbial suspensions were adjusted to 0.5 MacFarland and diluted 1:10 to yield 5*10⁵ CFU/ml and to each well, 0.005 ml of the bacterial inoculum was seeded. Control line with no bacterial inoculation and *P. aeruginosa* ATCC27853 was simultaneously maintained. Microplates were incubated aerobically at 37°C for 24 hours. The lowest concentration of the extracts that produced no visible bacterial growth was reported as the MIC. MBC values were determined using the first well showing no growth on media agar.

Results

Antibiogram results using disc diffusion method:

The results of Table 1 were obtained from 100 samples after performing antibiogram by disc diffusion method to determine the antibiotic resistance percentage of *Pseudomonas aeruginosa* in burn patients.

Determine the minimum inhibitory concentration (MIC) in 100 isolates of *Pseudomonas aeruginosa*:

Of the 100 isolates examined, 90% of the isolates were resistant to Maropenem, 7% to semi-sensitive and 3% susceptible. In addition, 90% of the isolates were resistant to Imipenem, 5% semi-sensitive and 5% sensitive. In the case of Ceftazidime, 72% of the isolates were resistant to Ceftazidime, 3% semi-sensitive and 25% sensitive, and 96% of ciprofloxacin were resistant, 1% sensitive and 3% sensitive. Based on the results, the antibiotic ciprofloxacin has the highest resistance and Ceftazidime has the least resistance among patients.

Table 1: Results of antibiotic resistance of *Pseudomonas aeruginosa* strains derived from diffusion disc.

Antibiotics	CIP	GM	IMP	MEM	DOR	PTZ	AN	CAZ	TC	CPM	PRL	ATM	CO
Resistance percent in burn patients	94	95	95	95	94	82	91	75	98	93	90	90	0

Table 2: Percentage of resistance and sensitivity of the samples to the antibiotics of Ceftazidime, meropenem, imipenem and ciprofloxacin based on MIC.

Antibiotics	resistant	semi-sensitive	sensitive
maropenem	90%	7%	3%
imipenem	90%	5%	5%
ceftazidime	72%	3%	25%
ciprofloxacin	96%	1%	3%

Table 3: The bacterial effect of acetone extract and methanolic extract of *Zataria multiflora* against *Pseudomonas aeruginosa* strains containing metallo- beta-lactamase enzymes.

<i>Zataria multiflora</i>		Concentration								
		25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.12 mg/ml	1.5 mg/ml	0.78 mg/ml	0.39 mg/ml	0.19 mg/ml	0.09 mg/ml
Acetone extract	MIC	-	75% (30)	25% (10)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	MBC	25% (10)	57.5% (23)	17.5% (7)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Methanol extract	MIC	-	77.5% (31)	22.5% (9)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	MBC	32.5% (13)	50% (20)	17.5% (7)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Table 4: The bacterial effect of acetone extract and methanolic extract of *Capsicum annum L.* against *Pseudomonas aeruginosa* strains containing metallo- beta-lactamase enzymes.

<i>Capsicum annum L.</i>		Concentration						
		6.25 mg/ml	3.12 mg/ml	1.5 mg/ml	0.78 mg/ml	0.39 mg/ml	0.19 mg/ml	0.09 mg/ml
Acetone extract	MIC	-	17.5% (7)	82.5% (33)	0.0%	0.0%	0.0%	0.0%
	MBC	12.5% (5)	37.5% (15)	50% (20)	0.0%	0.0%	0.0%	0.0%
Methanol extract	MIC	7.5% (3)	55% (22)	37.5% (15)	0.0%	0.0%	0.0%	0.0%
	MBC	17.5% (7)	57.5% (23)	25% (10)	0.0%	0.0%	0.0%	0.0%

Appropriate phenotypic testing to detect the metallo-beta-lactamase gene: After a phenotypic test, out of 100 samples, 81(81%) samples of *Pseudomonas* contained MBL. thus, in these samples, the diameter of the hole of the Imipenem / EDTA disk was 7 mm larger and larger than the diameter of the immortal field caused by the Imipenem disk alone.

Results of antimicrobial effects of total extract of three plants: Antibacterial potency of methanol and acetone of *Zataria multiflora*, *Capsicum annum L.*, *Piper nigrum L.* extracts against 40 isolates of *P.aeruginosa* containing MBL were evaluated by microdilution method as described by CLSI. MICs and MBCs (mg/ml) results of three plants against *P.aeruginosa* containing MBL were presented in Tables 3-5.

Table 5: The bacterial effect of acetone extract and methanolic extract of *Piper nigrum L.* against *Pseudomonas aeruginosa* strains containing Metallo- beta-lactamase enzymes.

<i>Piper nigrum L.</i>		Concentration								
		25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.12 mg/ml	1.5 mg/ml	0.78 mg/ml	0.39 mg/ml	0.19 mg/ml	0.09 mg/ml
Acetone extract	MIC	-	82.5% (33)	17.5% (7)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	MBC	37.5% (15)	55% (22)	7.5% (3)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Methanol extract	MIC	-	80% (32)	19% (8)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	MBC	32.5% (13)	50% (20)	17.5% (7)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Discussion

P. aeruginosa is a non-fermented Gram-negative bacterium, which is a common cause of nosocomial infections²². Therefore, due to the high mortality rate and injuries caused by this bacterium on burn patients due to defects in the immune system and skin lesions²³, in this study sought an effective disinfectant against this pathogen. In this study. Due to the resistance of the *P.aeruginosa* manufacturers of MBL to various chemical antibiotics, by the hydrolysis of almost all β -lactam antibiotics²⁵, it was assumed that herbal products could be a natural antibiotic to control and treat infection without endangering resistance.

P. aeruginosa was the most common bacteria in the last twenty years among burns patients hospitalized in Tehran hospitals. Our studies in parallel with most studies showed that strains containing MBL in this bacterium are resistant to almost all chemical antibiotics such as Meropenem, Imipenem, Ceftazidime, Amikacin, Tobramycin, Ciprofloxacin, Aztreonam, Piperacillin/Tazobactam, Ceftriaxone, Cefepime and Carbenicillin. Resistance mechanisms against antibiotics in MBL-containing strains in *P. aeruginosa* include 1) Reducing permeability of the extracellular membrane; 2) Efflux pump; 3) Converting to the effect of antibiotics; and 4) Degradation in the antibiotic structure²⁵. The prevalence of drug resistance to *P. aeruginosa* in burn wounds is reporting from other parts of the world. The higher prevalence of MBL in Tehran than the other countries, including the United States²⁶,

Spain²⁷, Korea²⁸ and India²⁹, is probably due to the length of hospitalization, the care conditions, and the use of antibiotics.

In the world as well as in Iran, the VIM beta-lactamase is the most dominant MBL type³⁰⁻³². Previous research in Iran showed that 11.43% of *P.aeruginosa* isolates from Tehran and 19.51% from Ahwaz (in Iran) were VIM-type positive.

In our study mortality rate due to infection with MBL producing *Pseudomonas* strains was 8.3% and in Brazil was 82.6%³⁰. Nowadays, carbapenems, especially imipenem and meropenem, are the main drugs in the treatment of *Pseudomonas aeruginosa*, with multiple resistances to antibiotics. Unfortunately, resistance to these antibiotics is also found in the strains it has arisen. The production of metallo-beta-lactamases that are transmitted horizontally through plasmids, as well as defects in outer membrane proteins, are among the main mechanisms for producing resistance to carbapenems³⁴. The present study supports the idea that *Zataria multiflora*, *Capsicum Annum L.* and *Piper nigrum L.* extracts might be useful as antimicrobial agents against *Pseudomonas aeruginosa* strains containing MBL. The methanolic and acetonic extract of all three plants have been shown to be effective in inhibiting the growth of MBL-containing *Pseudomonas aeruginosa*. In addition, from the extract of three herbs, the acetonic extract of *Capsicum Annum L.* at a concentration of 1.5 mg/ml has had the best effect in inhibiting this bacterium. In this study, the antimicrobial activity of these plants against *Pseudomonas aeruginosa* strains containing MBL

from hospital burn patients have been studied for the first time.

In 2013, Hashemi *et al*, reported the effects of *Zataria multiflora* and *Carum copticum* extract were studied in burn patients. According to the results, both plants at a concentration of 6.25mg/ml had a high effect on *Pseudomonas aeruginosa* containing MBL³⁵. Sharififar *et al*, showed that essential oil and methanol extract of endemic *Zataria multiflora* Boiss has an inhibitory effect on *S. aureus*, *E. coli*, *K. pneumoniae*, *S.epidermidis*, *E. faecalis*, *B. subtilis*, *S.typhi*, *S.marcescens* and *S.flexneri*³⁶. Furthermore, Mahboubi *et al*, showed that Staphylococcus growth can be inhibited by 0.5-1 µl/ml of *Zataria multiflora* Boiss essential oils. In addition, in a study by Javadian *et al*, the antimicrobial activity of hydroalcoholic extract of *Zataria multiflora* Boiss against *Pseudomonas aeruginosa* and *Staphylococcus aureus* resistant to antibiotics were investigated. The findings showed a small effect of the extracts with a decrease in their concentration. *Zataria multiflora* extract at the concentration of 10 mg/ml has the inhibitoriest effect on *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth, while the concentration of 20 mg/ml also had the most deleterious effect³⁷.

Conclusion

Resistance to common antibiotics in Tehran, especially in Motahhari Hospital was high. Physicians should carefully monitor the drug administration and send the specimen to the laboratory for antibiogram testing, in order to select the best drug for the patient. The results of this study indicate the presence of several mechanisms of resistance to beta-lactam antibiotics among *Pseudomonas aeruginosa* strains collected from burn patients. The emergence of these types of XDRs has led to health problems, especially in burn patients. According to the results, the methanolic and acetonic extract of all three plants have been shown to be effective in inhibiting the growth of MBL-containing *Pseudomonas aeruginosa*. In addition, from the extract of three herbs, the acetone extract of *Capsicum Annum L.* at a concentration of 1.5 mg/ml has had the best effect in inhibiting this bacterium.

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