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Original article

Antibacterial effects of *Quercus Brantii* fruits and *Stachys lavandulifolia* methanol extracts on imipenemase-type metallo-beta lactamase-producing *Pseudomonas aeruginosa*

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Abstract

Background and objectives: Metallobeta-lactamase production is one of the most important mechanisms of antibiotic resistance. The aim of the present study was to evaluate the antibacterial activity of *Stachys lavandulifolia* and *Quercus brantii* on the IMP-type metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. **Methods:** This study was performed on burn patients between January 2015 and November 2015. Susceptibility to the antibiotics and methanol extracts were evaluated by broth microdilution and disc diffusion methods. MBL-producing *P. aeruginosa* was detected by Combination Disk Diffusion Test (CDDT). The *bla* (VIM) and *bla* (IMP) genes detection were performed by PCR and sequencing methods. **Results:** Forty eight (57.9%) of 83 *P. aeruginosa* strains were resistant to imipenem and were *bla*IMP-1 genes positive, whereas none were *bla* (VIM) genes positive. In the hospitalized patients with MBL-producing *Pseudomonas* infection, the mortality rate was 4.48 (8.3%). It was found that *S. lavandulifolia* extract showed a high antibacterial effect on regular and IMP-producing *P. aeruginosa* strains at the concentration of 0.625 mg/mL, but *Q. Brandy* extract showed no antibacterial effect in the tested concentration. **Conclusion:** In burn patients MBL-producing *P. aeruginosa* has been found in high incidence. Detection of this *pseudomonas* and determination of drug resistance pattern is very important. The methanol extract of *S. lavandulifolia* showed suitable effects on MBL-producing *P. aeruginosa in vitro*; therefore, it could be suggested for further studies against carbapenem resistant *P. aeruginosa* isolates.

Keywords: metallo-beta-lactamases, *Pseudomonas aeruginosa*, *Quercus brantii*, *Stachys lavandulifolia*

Introduction

Pseudomonas aeruginosa is one of the leading nosocomial pathogens globally, which affects mainly immunocompromised hospitalized and burn patients. The clinical significance of this bacterium is mainly related to its multi resistance against antibacterial drugs and also becoming

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quickly resistant against the drugs during treatment [1]. Nosocomial infections related to this organism include wound infections in severe burn patients, catheter-associated urinary tract infections, ventilator-associated pneumonia and septicemia with their pathogenesis shown to be multifactorial. Acute infections such as, burn wound infections, sepsis, bacteremic pneumonia are associated with high mortality rates [2].

Nowadays increasing rate of antibiotic resistance among *P. aeruginosa* is a serious problem [3]. Carbapenems including doripenem, imipenem and meropenem are the main antimicrobial agents for treatment of *P. aeruginosa* infections [4].This class of antibiotic is classified as β-lactam antibiotics. Carbapenems has a wide range of activity and is the choice drug for treatment of *P. aeruginosa* infections, but carbapenem hydrolyzing enzymes such as MBLs are able to efficiently hydrolyze all betalactam antibiotics except monobactams [5]. The genes encoding MBLs that are located in integrons can be disseminated easily from one bacterium to another [6].

Many MBLs have been found in *P. aeruginosa*, including Sao Paolo Metallo-beta lactamase (SPM), Kyorin University Hospital Imipenemase (KHM), New Delhi Metallo-beta lactamase-1 (NDM-1) Imipenemase (IMP), Verona integron-encoded Metallo-β-lactamase (VIM), Seoul imipenemase (SIM), Australian Imipenemase (AIM) and German Imipenemase (GIM). Mobile elements inserted into class 1 integrons or plasmids often encode the genes of IMP and VIM-type in clinical isolates of *P.aeruginosa*, the distribution of which contributes to the outbreake of this mechanism of resistance [7]. MBLs production, which is related to genes like *vim* and *imp* is a common mechanism of resistance to carbapenems. The IMP-type enzymes hydrolyze penicillins, carbapenems and cephalosporins.These enzymes are among the clinically most important MBLs. MBLs such as IMP-1 belong to the molecular class B of Ambler and in group 3 of the Bush-Jacoby-Medeiros functional classification. IMP-1, emerged during the early 1990s in Japan and its variants were then detected in other countries [8,9].

The genus *Stachys* which belongs to the Lamiaceae family, consists of about 300 species widespread throughout the world. In Iran, 34 species of this genus are present among which, 13 are endemic [10]. *Stachys lavandulifolia* , with local name of "*Chaye-kuhi* " [11], has been traditionally used for headache, neuralgia, nervous conditions, as a tonic in dyspepsia, for healing wounds and skin inflammations and as astringent and antidiarrheal. In pharmacological studies *Stachys* species have shown anti-inflammatory, antibacterial, antinephritic and anxiolytic effects [12].

Oak (*Quercus*) is a predominant plant genus in central and northern Iran and includes many species. *Quercus*, which grows in the central forests of the country, is an important genus with 45 species; the predominant species is *Q. brantii* [13]. *Quercus brantii* is known as Persian oak, west oak or Zagros oak. It is native to the temperate regions of Asia particularly western Asia, including Iran, Iraq, Syria, and Turkey [14]. The Fruit of this plant is used for treating inflammation and chronic diarrhea [15]. Also extracts of *Q. brantii* have been shown to have pharmacological and biological properties such as wound-healing and antibacterial effects [16].

Our study was conducted on hospitalized burn patients in Shahid Motahari Hospital, Tehran, Iran, from January 2015 to November 2015. The aims of this study were to determine the antibiotic resistance patterns of *P.aeroginosa* and to detect *bla* VIM and *bla* IMP metallo-beta-lactamase genes, also to investigate the antimicrobial effects of *Q. brantii* fruits and *S. lavandulifolia* floweres methanol extracts on *P.aeroginosa* strains producing *bla*IMP isolated from the studied patients.

Experimental

Plant material

Flowers of *S. lavandulifolia* were collected from Ghorve Mountains (west of Iran) and fruits of *Q. brantii* plants were collected from Khorram Abad

Forest (west of Iran) in 2015. A voucher specimen was preserved at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran (Voucher Numbers 3511 and 3642 for *Q. brantii* and *S.lavandulifolia*, respectively).
The plant materials were washed and dried at room temperature and powdered using a mechanical grinder. Ten g of the dried materials were soaked in 100 mL of methanol for 72 h. Extracts were filtered through Whatman No. 1filterpaper and 0.45 micrometer membrane filter. Filtered extracts were concentrated in a vacuum evaporator.

Sampling
In 2015, hundred samples of *P. aeruginosa* was isolated from burn patients admitted to Shahid Motahari Hospital (level I burn care center), in Tehran (the research was approved by ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran in 2015). Samples were transferred in Stuart media and transported to the microbiology laboratory. Then cultured on Mac Conkey and Cetrimide agar (Merck, Germany) at 37 °C for 24 h. Colonies were identified, using catalase and oxidase tests, with growth at 42 °C and sugar fermentation test. Isolates of *P. aeruginosa* were stored at -70 °C in brain heart broth (Merck, Germany) with 20% glycerol [1].

Antimicrobial susceptibility testing
Antimicrobial susceptibility to amikacin (AK, 30 µg), tobramycin (TOB, 10 µg), gentamicin (GEN, 10 µg), piperacillin/tazobactam (PTZ, 100/10 µg), ciprofloxacin (CIP, 5 µg), cefepime (FEP, 30 µg), carbenicillin (Car,100 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg) and aztreonam (ATM, 30 µg) (Mast Group, UK) was tested on the isolated *P. aeruginosa* samples and the *P. aeruginosa* ATCC 27853 strain as the control by the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (Merck, Germany) based on Clinical Laboratory Standards Institute (CLSI) guidelines [17].

Minimum inhibitory concentration of antibiotics
MIC of different antibiotics was determined according to the CLSI guidelines by broth micro dilution method [17]. Briefly, the stock solutions of antibiotic powders (Himedia Co and GLAXO, England) were provided. The bacterial suspension equivalent to 0.5 McFarland standard was prepared. A 100 µL volume of each suspension was loaded into microtiter plate wells. Then 100 µL of stocks of each antibiotic were added and were incubated at 37 °C for 24 h.

Detection of MBL by phenotypic method
Meropenem and Meropenem + EDTA, Imipenem and Imipenem + EDTA discs were used to detect MBL-producing *P. aeruginosa*. MBL detection was performed by Combination Disk Diffusion Test (CDDT) [18].

Detection of blaIMP and blaVIM genes by PCR method
DNA was extracted using boiling method [19]. For detection of *blaIMP* and *blaVIM*, polymerase chain reaction (PCR) amplification was performed with primers VIM-F (5′ GTTTGGTCGCATATCGCAAC-3′) and VIM-R (5′-AATGCGCAGCACCAGGATAG-3′) and primers IMP-F (5′ GAAGGCGTTTATGTTCATAC-3′) and IMP-R (5′-GTATGTTTCAAGAGTGATGC-3′) under PCR conditions as reported previously [19].

Sequencing
Sequencing of forward strand was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed with Chromas 1.45 then BLAST in PubMed NCBI.

Microdilution test for extracts
The extracts were determined according to the method described by CLSI, 2012 the MIC of. *Quercus brantii* and *S. lavandulifolia* extracts

were diluted with 2% DMSO, the concentrations ranging from 0.078 to 10 mg/mL, were prepared. Muller Hinton broth was used as broth medium. After shaking, 0.1 mL of each extract was added to one well of 96-well microtiter plates. Microbial suspensions were adjusted to 0.5 McFarland and diluted 1:10 to yield 10^7 CFU/mL and 0.005 mL of the bacterial inoculum was added to each well. Control line with no bacterial inoculation and *P. aeruginosa* ATCC27853 were simultaneously maintained. Microplates were incubated at 37 °C for 24 h [1]. The test was carried out in quadruplicates for each extract. The lowest concentration of the extracts that produced no visible bacterial growth was reported as the MIC.

Results and Discussion

From 100 isolates of *P.aeroginosa* 83% were resistant to ceftazidime and imipenem. From 83 isolates that were resistant to imipenem, 48% produced metallo-beta-lactamase and all of them were resistant to cefepime, ceftriaxone, ampicillin, piperacillin-tazobactam, -meropenem, cefotaxime and ceftazidime; but 49% of *P. aeroginosa* isolates, were resistant to gentamicin. Distribution of antibiotics MICs for IMP-producing *P. aeruginosa* strains has been shown in table 1.

Table 1. Minimum inhibitory concentration (MIC) of different antibiotics for IMP-producing *Pseudomonas aeruginosa* strains

Antibiotics	**MIC(µg/mL)**					
	P.a IMP1	P.a IMP2	P.a IMP3	P.a IMP4	P.a IMP5	P.a IMP6
Ceftriaxone	≥256	≥256	≥256	≥256	≥256	≥256
Piperacillin/ Tazobactam	256	256	256	256	256	256
Ampicillin	≥256	≥256	≥256	≥256	≥256	≥256
Cefotaxime	≥256	≥256	≥256	≥256	≥256	≥256
Ceftazidime	≥256	≥256	≥256	≥256	≥256	≥256
Cefepime	128	128	128	128	128	128
Meropenem	64	32	64	64	64	64
Imipenem	128	128	128	128	128	128

P.a: *Pseudomonas aeruginosa*

The *blaVIM* gene was not detected in any of isolates while *blaIMP* was detected in 6 isolates by PCR method. Results of sequencing of *blaIMP* PCR product were confirmed to *blaIMP* in NCBI.

MICs results of *Q. brantii* and *S. lavandulifolia* extract on IMP-producing *P. aeruginosa* strains has been shown in table 2. The results of our study showed that the extract of *S. lavandulifolia* demonstrated considerable effects on regularand IMP-producing *P. aeruginosa* strains at the concentration of 0.625 mg/mL; however, the extract of *Q. brantii* was not active against the tested microorganisms in this study.

Table 2. Minimum inhibitory concentration (MIC) of *Stachyse lavandulifolia* and *Quercus brantii* extracts for IMP-producing *P. aeruginosa* strains

Strains of *P. aeruginosa*	***Stachyse lavandulifolia***	***Quercus brantii***
P.a IMP1	0.625mg/mL	10mg/ml<
P.a IMP2	0.312mg/mL	10mg/ml<
P.a IMP3	0.312mg/mL	10mg/ml<
P.a IMP4	0.312mg/mL	10mg/ml<
P.a IMP5	0.312mg/mL	10mg/ml<
P.a IMP6	0.625mg/mL	10mg/ml<
P. aeruginosa ATCC 27853	0.312mg/mL	10mg/ml<

P.a: *Pseudomonas aeruginosa*

With the increase in the indiscriminate use of antibiotics, the type and number of antibiotic resistant bacteria have raised and as a result the need for antibacterial drugs with new sources is peremptory [20].

Carbapenems are considered as drugs of choice for treatment of infections caused by multi drug resistant (MDR) Gram negative bacteria. Nowadays, the spread of carbapenem-resistant bacteria such as *P. aeruginosa* and *Acinetobacter baumannii* has become a major public health concern. The MBL producer *P. aeruginosa* are great problems in medical centers because they can hydrolyze all beta-lactam antibiotics and cause high mortality and morbidity rates [3].

The prevalence of MDR isolates has been increasing globally and become an important problem in hospitals [21]. In the present study, all of *P. aeruginosa* that produced MBL showed resistant to carbenicillin, ceftriaxone, aztreonam, tobramycin, ceftazidime, meropenem, amikacin, imipenem, ciprofloxacin, piperacillin/tazobactam

and cefepime; while 49% of isolates were resistant to gentamicin. Other antibiotic resistance mechanisms including efflux pumps, KPC enzymes and loss of the OprD (outer membrane porin) can also cause drug resistance [5].

Compared to other countries such as India [22], Korea [23], Spain [24], and USA [25], in our study MBLs were found in high prevalence because in our country's condition care, antibiotic utilization protocols and hospitalization duration is different. In studies in Tehran and Ahvaz VIM-positive *P. aeruginosa* were 11.43% and 19.51%, respectively. In this study IMP-1 producer *P. aeruginosa* was the main strain among the studied isolates, which is in accordance with other researches. Forty two isolates of *P. aeruginosa* were *bla* (IMP) and *bla* (VIM) negative and their antibiotic resistance may be related to other genes. Mortality related to MBL producing *P. aeruginosa* in the present study was 8.3%; however it was reported to be 82.6 %in Brazil [26].

The genes encoding Metallo-beta-lactamase spread easily on plasmids and cause nosocomial infections with high mortality rates [1]. Just a few drugs were active against MBL-producers *P. aeruginosa* that were isolated from Shahid Motahari Hospital, so it is necessary to change the way of treatment in order to prevent spread of resistance genes.

For obtaining new drugs, the use of plant resources can be considered valuable. In addition to clinical challenges of nosocomial infections, the emergence of resistant strains of bacteria and the side effects of antibiotics, explains the necessity for new antibacterial drugs especially with natural sources [20].

A wide range of plants have numerous healing properties and fewer side effects, therefore they can be used in the treatment of some diseases. *Quercus brantii*, Persian oak, is used for improving stomach and digestive problems by local people in the Zagros area, Iran. Tannin compounds in the plant have disinfectant and contractive effects. The extracts of *Q. brantii* have shown wound-healing and antibacterial effects [16].

S. lavandulifolia is a medicinal plant belonging to the *Labiatae* family. Flavonoids are found in large amounts in *S. lavandulifolia* and hypotensive, antihepatitis, antianxiety and antibacterial properties have previously been registered in pharmacological studies [27].

Biological activities of *S. lavandulifolia* and *Q. brantii* have been showed in many researches [10-15]; thus, in the present study their effects on IMP-type metallo-beta-lactamase-producing *P. aeruginosa* were evaluated. The results of our study showed that the methanol extracts of *S. lavandulifolia* inhibited the growth of IMP-type metallobetalactamase-producing *P. aeruginosa* at the concentration of 0.625 mg/mL which might be a promising approach for developing new anti-*pseudomonas* drugs. On the other hand, the methanol extract of *Q. brantii* fruits was not effective on IMP-type metallo-beta-lactamase-producing *P. aeruginosa*.

Our study was the first report on antibacterial activity of *S. lavandulifolia* and *Q. brantii* extracts on IMP-type metallo-beta-lactamase-producing *P. aeruginosa*. *Stachys lavandulifolia* extract was able to establish inhibition of growth with MIC 0.312 mg/mL and 0.625 mg/mL, while MIC of *Q. brantii* for IMP-producing *P.aeruginosa* strains was 10> mg/mL and showed no considerable effect on these strains.

In 2009, Safary *et al.* reported that *Salmonella typhi, Proteus mirabilis, Shigella dysenteriae* and *P. aeruginosa* growth could be inhibited by the use of 0.4-0.5 g/mL of *Q. brantii* extract, but in our study *Q.brandy* extract showed no antimicrobial effect on the MBL-producing *P. aeruginosa* [28].

Sadeghian *et al*. reported that antibacterial effect of the *Q. brantii* ethanol extract on *E.coli* was significant, but there was no effect found on *H.pylori* [13]. Furthermore, Fooladvand *et al*. have showed the antibacterial activity of *S. lavandulifolia* Vahl against *against Proteus mirabilis,* and *Enterococcus faecalis.*. The MIC concentrations ranged from 25 to 100 ppm [29].

Also in a study conducted by Bahar *et al*., *Q. brantii* subsp. *persica* showed inhibition of bacterial growth at concentrations between 0.2 and 1.2 mg/mL. The mean of biofilm inhibition by *Q. brantii* at a MIC50 in, *E. coli* and *P. aeruginosa* were 45% and 35%, respectively [10].

The results of previous studies have shown that methanol extracts have significant activity [30], which were confirmed in our study, also in the research carried out by Shahnam *et al*., methanol extract of *S. lavandulifolia* showed more inhibitory effects against *Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus agalactiae* [31].

Herbal medicines with good efficiency can be suitable substitutes for antimicrobial drugs; however, further investigations are necessary.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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