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The Study of the Inhibition Effects of *Satureja khuzestaniea* Essence on the Gene Expression of bla-$^{OXA-23}$ in Multidrug-Resistant Strains of *Acinetobacter baumannii*

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Abstract

Background: *Acinetobacter baumannii* is one of the Gram-negative bacteria in nosocomial infections that is resistant to treatment and control. The isolation of these bacteria is increasing significantly throughout the world. With increasing populations and a reduction in the use of synthetic drugs, many of these medicinal herbs are replacing other treatments.
Objectives: The present study aims to investigate the inhibitory effects of *Satureja khuzestaniea* essence on the expression of the antibiotic resistance genes bla-$^{OXA-23}$ and Housekeeping DNA gyrase-A in multidrug-resistant strains of *Acinetobacter baumannii*, using the RT-PCR technique.
Methods: *Satureja khuzestaniea* essential oil was collected from the Barij essence research farm in May 2010. Five multidrug-resistant strains of *Acinetobacter baumannii* that contain the bla-$^{OXA-23}$ and housekeeping DNA gyrase-A genes were selected. The disk diffusion method was used to evaluate the antimicrobial effect of essential oil, and major components were used. The minimum inhibitory concentration (MIC) values of the *Satureja khuzestaniea* essential oil were determined via a broth macro dilution assay, as recommended by the CLSI. Finally, an analysis of the bla-$^{OXA-23}$ and Housekeeping DNA gyrase-A was performed using the RT-PCR technique.
Results: The major components of *Satureja khuzestaniea* essence are carvacrol (90.88%), ρ-cymene (3.11%), γ terpinene (1.24%), and linalool (0.91%). The RT-PCR technique demonstrates that *Satureja khuzestaniea* with an MIC of 0.2 μL/mL has the effect of reducing the expression of the antibiotic resistance gene bla-$^{OXA-2}$. The essence has no inhibitory effect, however, on the Housekeeping DNA gyrase-A gene.
Conclusions: This study indicates that *Satureja khuzestaniea* essence has inhibitory effects on the gene expression of antibiotic resistance in bla-$^{OXA-23}$ that has a high MIC. Given that this essence has a good inhibitory effect on gene expression in MDR *Acinetobacter baumannii* and bla-$^{OXA-23}$, the results indicate that it could be used as a natural way to prevent the growth of *Acinetobacter baumannii*.

Keywords: RT-PCR, bla-$^{OXA-23}$, *Satureja khuzestaniea*

1. Background

Acinetobacter baumannii is a Gram-negative bacillus that is an aerobic, pleomorphic, non-motile, non-fermenting, and opportunistic pathogen (1). Infections from these bacteria, particularly in patients in intensive care units in hospitals, are very dangerous (2). People with cystic fibrosis, neutropenia, immune deficiency, or a breach of the treatment and immunity barriers that normally prevent bacteria from attack are all at risk of being infected with *Acinetobacter baumannii* (3). This bacterium causes infections of the respiratory tract, bloodstream, skin, and soft tissues (4). *Acinetobacter baumannii* is very resistant to antimicrobial agents; the resistance may be intrinsic or obtained via genetic factors (5). Most strains of *Acinetobacter baumannii* are resistant to Ampicillin, Amoxicillin-Clavulanic acid, Penicillin-resistant staphylococcal, broad-spectrum cephalosporins (except Ceftazidime and Cefepime), Tetracycline, Macrolides, Rifampin, and Chloramphenicol (6).

The most common and serious MDR pathogens are encompassed within the acronym "ESKAPE," which stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (7). The pathogenic potential of *Acinetobacter baumannii* includes the ability to adhere to surfaces and material from unrelated genera, making it a versatile bacterium and a difficult adversary to control and

eliminate (8). Carbapenem resistance to *A. baumannii* is due to a variety of combined mechanisms, such as hydrolysis by beta-lactamases, alterations in outer-membrane protein, penicillin-binding proteins, and the increased activity of efflux pumps (9). Carbapenem-hydrolyzing bla-$^{OXA-23}$ was first discovered in *A. baumannii* in 1985. Since then, several cases have been reported around the world (10). An acquired resistance to carbapenems is mediated by Ambler class D beta-lactamases or OXA-type carbapenamases, as well as Ambler class B metallo-beta-lactamases that are encoded by transmissible genes, which account for most of the resistance to carbapenems (5, 11). The use of medicinal herbaceous drugs in treatments has been recommended since ancient times. Humans have noted their beneficial effects and utilized them (12). With increasing populations and urban growth, as well as a reduction in the use of synthetic drugs, many of these medicinal herbs have begun to be used more frequently. Usage problems such as the increasing resistance of microorganisms to such herbs and the diminishing effectiveness that accompanies continued application have been reported.

2. Objectives

The present study aims to investigate the inhibitory effects of *Satureja khuzestaniea* essence on the expression of the antibiotic resistance genes bla-$^{OXA-23}$ and Housekeeping DNA gyrase-A in multidrug-resistant strains of *Acinetobacter baumannii*, using the RT-PCR technique.

3. Methods

Satureja khuzestaniea was collected in Khoramabad, Iran, in 2013. The plant was cut into small pieces (100 g) and subjected to hydro distillation for 6 hours, using Clevenger-type apparatus. The oil was obtained and dried using anhydrous sodium sulfate. It was then stored in tightly closed, dark vial. Oil analysis was carried out using GC and GC/MS. The GC apparatus was an Agilent technology (HP) 6890 system, with a HP-5MS capillary column (60 m .0.25 mm i.d., film thickness 0.25 mm). The initial oven temperature was 40°C a temperature that was retained for 1 minute, before being increased to 23°C at a rate of 31°C/min. The higher temperature was then kept constant for 10 min. Helium was used as the carrier gas, at a flow rate of 1.0 mL/min. The detector and injector temperatures were 25°C and 23°C, respectively. GC/MS analysis was conducted via a HP 6890 GC system, together with a 5973 network mass selective detector (with a capillary column that was the same as that mentioned above) and carrier gas helium with a flow rate of 1 mL/min (with a split ratio equal to 1/50). The injector and the oven-temperature programming were identical to those used for GC. The compounds in the oil were identified via a comparison of their retention indices (RI) and mass spectra fragmentation with those stored on the Wiley 7n.1 mass computer library and at NIST (National Institute of Standards and Technology) (13).

3.1. Microbial Strains

Five multidrug-resistant strains of *Acinetobacter baumannii* (among seventy-five strains of *Acinetobacter baumannii* containing bla-$^{OXA-23}$ found in Tehran's hospitals) were selected and were approved for use in this study via the PCR technique. The bacterium was isolated from clinical specimens such as wound swabs, CSF (cerebra spinal fluid), respiratory tract secretions, blood cultures, and urine. *Acinetobacter baumannii* ATCC 19606 was employed in this study as a model reference strain.

3.2. Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed on different classes of antibiotics, following the Clinical Laboratory Standard Institute (CLSI) guidelines (5). Antibiogram testing was performed on white amikacin (30 μg), Oxacillin (30 μg), Kanamycin (30 μg), Gentamicin (10 μg), Neomycin (30 μg), and imipenem (10 μg). The antimicrobial activity of *Acinetobacter baumannii* in *Satureja khuzestaniea* essence was studied using a disk diffusion method (11). Here, 10 μL from essence was dissolved in 90 μL DMSO. The final concentration was about 0.1 (mg/mL). Briefly, 0.1 mL of this bacterium (1.5×10^{8} CFU /mL) was spread on Mueller-Hinton Agar plates. Next, 6 mm sterile discs containing 3 μL (concentration of about 0.1 (mg/mL) of essential oil were laid on the bacterial culture media. Inhibition zone diameter discs containing essential oil in microbial cultures were measured after the incubation time, and the results were reported in mm. The concentrations of essential oils for each bacterial sample were repeated three times and the average diameters were documented. Disks containing 3 μL of dimethyl sulfoxide were used as negative controls.

3.3. Determining the Amount of the Minimum Inhibitory Concentration (MIC)

MIC values were determined using a broth macro dilution assay, as recommended by the CLSI (11). To determine the MIC for *Satureja khuzestaniea* essential oil, 10 μL of the oil was dissolved in 90 μL DMSO. The final concentration was about 0.1 (mg/mL). Broth samples were cultured in tubes containing 5 mL Mueller-Hinton, which were incubated at 37°C for 18 - 24 hours. After incubating each sample, five tubes containing 1ml Mueller-Hinton broth were

prepared. To each of the tubes, 1, 2, 3, 4, and 5 μL of essential oil was added, respectively. After mixing, a bacterial suspension was added to make a final volume 5×10^6 CFU/mL. The samples were then incubated at 37°C for 18 - 24 hours and the results were evaluated. In this series of experiments, a positive control tube (without oil) was also used. The bacterial growth was assessed visually after the incubation period. Any turbidity or clear slights were considered signs of resistance. The minimum concentration of bacteria produced no significant growth, as the MIC was determined. MBC values were the first tube that showing no growth on solid media.

3.4. Detection of the Gene bla-OXA-23 via the PCR Technique

Genomic bacterial DNA was extracted from the five strains using the boiling and freezing method. The bacterial samples were boiled at 95°C for 5 minutes and then frozen at 0°C for 5 minutes in a final volume of 50 μL distilled sterile water. These actions were repeated three times. After centrifugation at 13000 g, the supernatants were used as DNA templates.

Allele ID software was used for the primer design. PCR was performed in standard enzyme Taq DNA polymerase. The primers used to amplify the target fragment were bla-$^{OXA-23}$ Forward (5'- CACTAGGAGAAGCCATGAAG < C > -3'), reverse (5' CAGCATTACCGAAACCAATAC < G > -3'), and DNA gyrase-A Forward (5'- AAGGCCGTCCAATCGTGAA < T > -3'), Reverse (5'-AACCGTACCAGAAGCTGTC < G > -3'). The PCR reactions were performed with a Cinnagen kit in a final volume of 25 μL, containing 12.5 μL Master Mix (1 x) and 5 μL of DNA extract (20 pg), 1 μL F Primer (0.1 - 1 μM), 1 μL R Primer (0.1 - 1 μM), and 5.5 μL Sterile Deionized Water. The cycles for the bla-$^{OXA-23}$ gene mixtures were incubated as follows: primary denaturation for 120 seconds at 95°C, secondary denaturation for 45 seconds at 95°C, annealing for 60 s at 53°C, and an extension of 30 seconds at 72°C, followed by a final extension of 90 seconds at 72°C. For DNA gyrase-A, the gene mixtures were amplified through step primary denaturation for 120 seconds at 95°C, secondary denaturation for 5 seconds at 95°C, annealing for 60 seconds at 51.8°C, and an extension of 30 seconds at 72°C, followed by a final extension of 90 seconds at 72°C. Thirty-five cycles were performed. The final products were analyzed using electrophoresis on 1% agarose gel (Cinnagen) containing 0.1 g of ethidium bromide per mL, in a TAE (Tris-Acetate EDTA) buffer. The PCR product was visualized under UV light and was also photographed.

3.5. Analysis of bla- OXA-23 With the RT-PCR Technique

The total mRNA was extracted from five isolates of *A. baumannii*. In order for quantitative evaluation to take place, the product obtained from the RNA extraction was measured using a spectrophotometer at a wavelength of 260 nm. A nano-drop spectrophotometer device following the principles and high levels of precision in the measurement of concentrations of nucleic acids was used to measure the concentration of the extracted RNA. For each sample, the cDNA was synthesized and the alterations in the expression levels of the bla-$^{OXA-23}$ and gyrA genes were identified using the RT-PCR method (Cinnagen), under the following conditions: 5 μL of DNase-treated RNA was placed in a final volume of 25 μL, containing 50 mM Tris-HCl pH 8.3, 75 Mm KCl, 3 mM $MgCl_2$, 10 mM DTT, 400 μM of each nucleotide, 1 μM of the reverse primer, 1 μM of the forward primer, and 100 U M-MLV reverse transcriptase. The reactions were incubated for 1 hour at 42°C, followed by a 5 minutes incubation at 95°C. 10 μL of the c DNA was used for the amplification of specific bla^{OXA-23} mRNA and DNA gyrase -A. For the bla^{OXA-23} m RNA, 120 seconds initial denaturation was carried out at 94°C, followed by thirty-five cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 53°C, and a 30 seconds extension at 72°C, with a final extension step for 5 minutes at 72°C. For the DNA gyrase-A mRNA, 120 seconds initial denaturation was carried out at 94°C, followed by thirty-five cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 51.8°C, and a 30 seconds extension at 72°C, with a final extension step of 5 minutes at 72°C. Both PCR products were detected in 1% agarose gel. Each RT-PCR test was performed in triplicate.

4. Results

The major components identified were carvacrol (90.88%), ρ-cymene (3.11%), γ terpinene (1.24%), linalool (0.91%), Terpinene-4-01 (0.91%), α-Terpinene (0.49%), β-Myrcene (0.39%), Borneol (0.35%), and α-Pinene (0.28%). The antibiotic susceptibility test results illustrate the levels of resistance of the five *Acinetobacter baumannii* strains are as follows: Oxacillin (100%), Amikacin (75%), Kanamycin (68%), Gentamicin (60%), Imipenem (60%), and Neomycin (89%). The inhibitory effects of *Satureja khuzestaniea* on drug-resistant strains are detailed in Table 1.

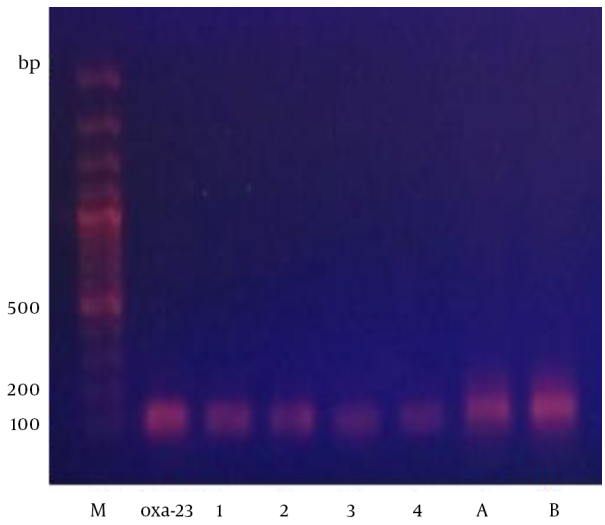
The RT-PCR results before and after bacteria treatment revealed that the expression of the bla- $^{OXA-23}$ gene was reduced significantly in the presence of *Satureja khuzestaniea* extract (Figure 1), even though this gene was expressed highly in this herb before its exposure to the bacteria. As expected, the expression of gyrA gene was relatively constant across the samples and controls. The expression of the gyrA gene served as an internal control, ensuring that equal amounts of RNA were used in the RT-PCR tests. Surprisingly, the expression levels of the bla-$^{OXA-23}$ gene decreased in the presence of *Satureja khuzestaniea*. However,

Table 1. MIC Results (μg/mL) for Satureja Essence and Zone of Growth Inhibition (mm) for *Acinetobacter baumannii*

Pathogenic Strains	*Satureja khuzestaniea* Essence			Antimicrobial Agents MIC, μg/mL Zone of Growth Inhibition, mm					
A. baumannii	MIC, μg/mL	MBC, μg/mL	Zone of Growth Inhibition, mm	CIP	GM/IMP	AMK/CRO/ OX/K/N	CIP	GM/IMP	AMK/CRO/ OX/K/N
A/3	0.2	0.4	35	> 4	> 16	> 64	0	0	0
A/64	0.3	0.5	24	> 4	> 16	> 64	0	0	0
A/69	0.5	0.7	30	> 4	>16	> 64	0	0	0
A/75	0.2	0.4	25	> 4	> 16	> 64	0	0	0
A/77	0.2	0.4	15	>4	> 16	> 64	0	0	0

the expression of the DNA gyrA gene that was used as an internal control did not change before and after the treatment took place.

Figure 1. Agar Gel Electrophoresis Results for RT-PCR with bla^{OXA23} and DNA gyrase-A in the Presence of the *Acinetobacter baumannii* Strain, Before and After the Face With Essential Oils



(Oxa-23) *A. baumannii* strain bla^{OXA23} gene before the face with essential oils, (1, 2, 3, 4) *A. baumannii* strain after the face with essential oils, A, DNA gyrase A gene before the face with essential oils; B, DNA gyrase A gene after the face with essential oils.

5. Discussion

Currently, nospresent study indicates thatocomial infections caused by multidrug-resistant strains of *Acinetobacter baumannii* (MDR-AB) are among the most difficult to treat, and they continue to present serious challenges to clinicians' empirical and therapeutic decisions in relation to burned patients (14). The increasing prevalence of XDR and PDR*A. baumannii* strains and the limited treatment options have prompted the use of antibiotic combinations such as Tigecycline and Colistin as therapeutic regimens (15, 16). Resistant strains of bacterial pathogens are a major source of high morbidity, mortality, and increased cost, making their treatment much more difficult (11). Traditionally, plants have played an important role in the treatment of diseases; as such, they may be considered sources of drug therapies that could be used instead of carbapenem. Nosocomial infections due to multidrug-resistant *Acinetobacter baumannii* are more resistant to treatment than other nosocomial infections, and have led to many economic and health-related problems in society (17). In addition to the high mortality rates associated with such infections, the cost of treating them is high (18). It is well known that the bla-$^{OXA-23}$-like genes are some of the most prevalent acquired carbapenemase-encoding genes worldwide. They can exist on chromosomes or plasmids in different genetic structures (19). The present study indicates that *Satureja khuzestaniea* essence has inhibitory effects on the gene expression of the antibiotic resistance of bla-$^{OXA-23}$ with a low MIC, and also has inhibitory effects on MDR *Acinetobacter baumanii*.

This research indicates, therefore, that the *Satureja khuzestaniea* essential oil could be used as a complementary therapy or a replacement for carbapenem, which is a β-lactam. In this study, we also identified a compound named carvacrol, which has good antibacterial activity against *A. baumannii*, with an MIC of 0.2 μL/mL.

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Footnote

Authors' Contribution: Hossein Saghi: research conceptualization and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and critical revision of the manuscript's intellectual content; Ferdoes Amiri Dastjerdi, Bahador Zahedi, Mahmoud Khorrami, Majid Efati: administrative/technical/material

support and study supervision; Davoud Esmaeili: research conceptualization and design, critical revision of the manuscript's intellectual content, statistical analysis, administrative/technical/material support, and study supervision.

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