

Reduced Efflux Pumps Expression of *Pseudomonas Aeruginosa* with *Satureja Khuzistanica* Essential Oil

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What's Known

- *Satureja khuzistanica* essential oil (SKEO) has antibacterial activity against *Pseudomonas aeruginosa*.

What's New

- For the first time, the effect of *Satureja khuzistanica* essential oil on the expression of efflux pump genes of *Pseudomonas aeruginosa* is demonstrated. *Satureja khuzistanica* essential oil reduced the expression of efflux pumps and the MIC values of norfloxacin and gentamicin *in vitro*.

Abstract

Background: Efflux pumps such as MexEF-OprN and mexXY-OprM play an important role in the resistance of *Pseudomonas aeruginosa* (*P. aeruginosa*) to antibiotics. The present study aimed to assess the reduced expression of efflux pump genes of *P. aeruginosa* with *Satureja khuzistanica* essential oil (SKEO).

Methods: The present cross-sectional study was conducted in 2016 at the Microbiology Laboratory of Baqiyatallah University of Medical Sciences, Tehran, Iran. The disk diffusion method was used for susceptibility testing of gentamicin and norfloxacin. Minimum inhibitory concentration (MIC) was determined for gentamicin and norfloxacin. The antibacterial efficacy of SKEO was defined by determining the MIC values using the microdilution method. *In vitro*, the synergistic interaction of SKEO combined with gentamicin or norfloxacin was examined via checkerboard assay and defined as a fractional inhibitory concentration index. The reverse transcription-polymerase chain reaction technique was used to measure changes in the expression of the efflux pump genes. The data were analyzed using SPSS software version 16.0, and $P < 0.05$ was considered statistically significant.

Results: The MIC values of SKEO were in the range of 6 to 12 $\mu\text{g/mL}$. In the presence of sub-inhibitory concentrations (1.16 to 2 MIC) of SKEO, synergistic effects were revealed using the checkerboard method. The effect of norfloxacin and gentamicin increased up to 8-fold. The expression of *mexY* and *mexE* was reduced after treatment with SKEO.

Conclusion: SKEO reduced the expression of efflux pumps and the MIC values of norfloxacin and gentamicin *in vitro*.

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Keywords • Efflux pump • Gene expression • *Pseudomonas aeruginosa* • Norfloxacin • Gentamicin

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an important cause of nosocomial infections, which can rapidly develop resistance to many antibiotics through various mechanisms such as the high expression of efflux pumps.¹ Resistance-nodulation-division family transporters are the main category of efflux pumps in *P. aeruginosa* and contain at least 10 different types of efflux pumps.² MexXY-OprM and MexEF-OprN are the two most relevant pumps systems involved in resistance to antimicrobial

agents.³ MexXY-OprM has a main role in extruding various antibiotics in *P. aeruginosa* isolates, particularly beta-lactam antibiotics.⁴ Its expression commonly occurs in wild type strains and involves innate bacterial resistance to a number of antibiotics.^{2, 6} However, MexEF-OprN pump is expressed at low levels in wild-type isolates^{7, 8} and contributes to acquired resistance to antibiotics.⁹

A previous study described some compounds such as herbal extracts could inhibit the activity of efflux pumps.¹⁰ When plant extracts are used concurrently with chemical drugs, they enhance the effectiveness of antibiotics and can also act as an efflux pump inhibitor (EPI).¹¹ EPIs interrupt the extruding process of antibiotics by interference with the regulatory steps thereby suppressing the efflux pumps gene expression.¹² Checkerboard and real-time polymerase chain reaction (PCR) techniques can be used to assess the indirect activity of essential oils on the gene expression of efflux pumps. Compared with the checkerboard method, real-time PCR is a rapid technique with a high specificity and sensitivity, low contamination rate, and excellent efficiency in determining the synergistic effect.¹³

Satureja khuzistanica (*S. khuzistanica*) is an endemic plant in southern Iran. It has an antimicrobial activity due to the presence of essential oil content.¹⁴ The principal components of *S. khuzistanica* are thymol and carvacrol, which can damage bacteria. These substances, after disruption of efflux pumps, indirectly inhibit the gene expression of efflux pumps. The present study aimed to assess the reduced expression of efflux pump genes of *P. aeruginosa* with *S. khuzistanica* essential oil (SKEO).

Materials and Methods

The present cross-sectional study was conducted in 2016 at the Microbiology Laboratory of Baqiyatallah University of Medical Sciences, Tehran, Iran. The minimum inhibitory concentration (MIC) and checkerboard synergy assays were carried out in the cation-adjusted Mueller-Hinton broth (Hi-Media, India).

Strains and Inoculum Preparation

P. aeruginosa strains were isolated from the pus and wound specimens of burn patients referred to Shahid Motahari Hospital, Tehran, Iran. Multidrug-resistant (MDR) isolates were detected using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, supplement M100) instructions. *P. aeruginosa* PAO1 was used as a standard strain.¹⁵

Preparation of SKEO

Based on the method recommended by the European Pharmacopeia, dry aerial parts (100 g) of *S. Khuzistanica* were subjected to hydrodistillation for three hours using a Clevenger-type apparatus (Bakhtaran Co., Iran). The content of the device was kept at room temperature for 24 hours and subsequently mixed at the speed of 130 rpm using a shaker. A rotary device was used to remove solvents from the samples. Then, the sample was filtered using a vacuum pump. The obtained extract was weighed and then dissolved in dimethyl sulfoxide (DMSO) solvent (Merck, Germany). The solution was kept in a refrigerator (Donar, Iran) at 4 °C for further use.

Determination of MIC

The macro broth dilution method was used to determine the MIC value of SKEO against MDR isolates. To dissolve SKEO, 100 µg/mL DMSO solvent was added to SKEO concentrations of 100, 120, 140, 160, 180, 200, 220, and 240 µg in eight sterile glass tubes (13×100 mm). Each tube was filled with 1 mL of Muller-Hinton broth and isolates were inoculated into tubes. After mixing, 1 mL of bacterial suspension (10⁶ CFU/mL) was added to each tube and the final concentration of 5×10⁵ CFU/mL was obtained. After incubation at 37 °C for 24 hours, the minimal concentration of SKEO inhibiting bacterial growth was defined as MIC.¹⁶

RNA Extraction and cDNA Synthesis

A total of five MDR isolates were cultured in Muller-Hinton broth and suspended in diluted SKEO with a concentration below the MIC value and incubated at 37 °C for 24 hours. The total RNA was extracted using an RNA extraction kit (Qiagen, USA) according to the manufacturer's instruction. Then, cDNA was synthesized in a total volume of 20 µL reaction mixture, including 2 µg of the extracted RNA, 1 µL of deoxyribonucleotide triphosphate (10 mM), 50 ng of random Hexamer primer, 2 µL of M-MuLV buffer (10X) (Macrogen®, Korea), and 100 U of M-MuLV reverse transcriptase under an initial denaturation at 65 °C for five minutes and reverse transcription steps at 42 °C for one hour (Vivantis Technologies, Malaysia). The products were then used in the reverse transcription-polymerase chain reaction (RT-PCR) and PCR assay. The primers used in the PCR, RT-PCR, and quantitative reverse transcription PCR (qRT-PCR) assays are presented in table 1.

The RT-PCR reaction was performed using the RevertAid first-strand cDNA synthesis kit (Fermentas, USA) in a total volume of 25 µL

Table 1: Oligonucleotides sequences used in the present study

Primers	Sequence (5'→3')	Amplicon size (bp)	Reference
<i>mexY</i> -F	GCCCAACGACATCTACTTCA	108	Present study
<i>mexY</i> -R	ATGCCTTCCTGGTAATGGTC		
<i>mexE</i> -F	TCCTCAAGTACGTCGAGCTG	131	Present study
<i>mexE</i> -R	GACCTGGTTGTCGAGGAAGT		
<i>gyrA</i> -F	GGTCTGGGCATAGAGTTGT	121	Jalalvandi ¹⁷
<i>gyrA</i> -R	GAAGATCGAGGGTATTCCG		

reaction mixture, including 1 µg of cDNA (10 mM) and 0.3 pmol of each primer (table 1). The PCR condition included an initial denaturation at 95 °C for two minutes; 35 cycles at 95 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 30 seconds; and a final extension at 72 °C for five minutes. *GyrA* was used as an internal control. PCR products were subjected to 1% agarose gel electrophoresis and visualized under Ultraviolet (UV) light using the gel documentation system (Bio-Rad, Germany).

Real-Time PCR

The changes in the expression level of *mexE* and *mexY* genes before and after being subjected to SKEO were identified through cDNA amplification using the qPCR method. Real-time PCR was performed using the Corbett Palm-Cycler gradient thermal cycler (Qiagen, Germany) under the following conditions: 12.5 µL of Maxima SYBR Green/ROX qPCR Master Mix 2X (Fermentas, USA), 2 µL of template, and 0.3 pmol of each primer (table 1). Amplification of all genes was carried out with one cycle pre-denaturation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 58 °C for 20 seconds, and elongation at 72 °C for 20 seconds, followed by a final step at 72 °C for 60 seconds. Melting curve analysis was drawn at the terminal of the 40th cycle between 58-95 °C. Moreover, the Livak method was used to determine relative changes in the expression of *mexE* and *mexY* genes in comparison with the housekeeping genes.

Determination of MIC and Checkerboard Synergy Assay

The MIC values of gentamicin (MAST, England) and norfloxacin (MAST, England) in the absence and presence of SKEO were characterized using macro broth dilution and checkerboard assays. To assess the synergistic effects of SKEO, two-fold serial dilutions of gentamicin and norfloxacin were prepared and added into a 96-well microtitre plate. Dilutions for each antimicrobial agent ranged from 1.16 dilution to 2 sub-inhibitory concentrations. The second agent (SKEO) was also serially diluted

at different sub-inhibitory concentrations, and 50 µL of each dilution was added into each well. Then, 100 µL of the bacterial suspension (10⁶ CFU/mL) was added into the wells with a final volume of 200 µL. Each experiment was controlled using solvent and growth sterility tests. All tests were carried out in duplicate. The microtiter plates were incubated at 37 °C for 18 hours under aerobic conditions. Then, 10 µL from each well was poured on the Muller-Hinton agar, and the checkerboard synergy result was obtained by calculating the fractional inhibitory concentration index (FICI) with the following formula:¹⁸

$$\frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}} + \frac{\text{MIC of SKEO in combination}}{\text{MIC of SKEO alone}}$$

Based on the FICI value, synergism was defined as FICI ≤ 0.5, indifferent as 0.5 < FICI ≤ 4, and antagonism as FICI > 4.

Statistical Analysis

The data were analyzed using SPSS software version 16.0. One-way analysis of variance (ANOVA) was used to compare the means of three or more groups. The *post hoc Tukey HSD test* was used for pairwise comparisons of the groups. P < 0.05 was considered as statistically significant.

Results

Antibacterial Effect of SKEO, Gentamicin, and Norfloxacin

The MIC values of gentamicin, norfloxacin, and SKEO against PAO1 and five MDR strains were investigated. As shown in table 2, SKEO showed dose-dependent antibacterial activity against *P. aeruginosa* strains. The MIC values of SKEO were in the range of 6 to 12 µg/mL and the PAO1 was the most sensitive strain.

Effects of Antibiotics and Extract Combination

The effect of SKEO combined with gentamicin or norfloxacin was evaluated by checkerboard synergy assay. As shown in table 2, based on the FICI value, the results were categorized as synergism and indifferent (antagonism).

Table 2: Antibacterial activity of *Satureja khuzistanica* essential oil combined with antibiotics, and the results of synergy tests via the checkerboard method

Strains	MIC (µg/mL)			FICI of SKEO with antibiotics	
	SKEO	Gentamicin	Norfloxacin	Gentamicin	Norfloxacin
PAO1	6	2 (S)	0.5 (S)	2 (I)	1.83 (I)
PA9	10	4096 (R)	32 (R)	1.1 (I)	0.32 (Sy)
PA11	8	4096 (R)	16 (R)	1 (I)	0.37 (Sy)
PA13	9	>4096 (R)	16 (R)	ND	0.45 (Sy)
PA41	7	2048 (R)	16 (R)	0.928 (I)	0.41 (Sy)
PA42	12	>4096 (R)	64 (R)	ND	0.83 (I)

I: Indifferent, Sy: Synergism, S: Sensitive, R: Resistance, ND: Not determined, SKEO: *Satureja khuzistanica* essential oil, MIC: Minimum Inhibitory Concentration, FICI: Fractional Inhibitory Concentration Index

Table 3: Quantification of the expression levels of normalized *mexE* and *mexY* genes by quantitative polymerase chain reaction and minimum inhibitory concentration of *Satureja khuzistanica* essential oil against multidrug-resistant isolates

Isolates	<i>mexE</i> expression (before)	<i>mexY</i> expression (before)	Total expression of both genes (before)	MIC of SKEO (µg/mL)	<i>mexE</i> expression (after)	<i>mexY</i> expression (after)
PA9	5.55±0.03	1.18±0.08	6.73±0.05	10	1.12±0.02	0.03±0.008
PA11	1.87±0.06	1.24±0.04	3.02±0.01	8	0.45±0.03	0.04±0.005
PA13	1.67±0.01	3.88±0.05	3.02±0.01	9	0.49±0.02	0.07±0.002
PA41	1.77±0.02	0.54±0.02	2.31±0.03	7	0.42±0.06	0.01±0.009
PA42	10.04±0.06	2.75±0.06	12.79±0.09	12	1.97±0.02	0.05±0.001
Mean	4.18±0.03	1.92±0.05	6.08±0.04	9.2	0.89±0.03	0.04±0.005

MIC: Minimum inhibitory concentration, SKEO: *Satureja khuzistanica* essential oil

Treating the MDR strains of *P. aeruginosa* to SKEO with antibiotics showed a FICI value between 0.37 and 0.45, indicating a synergistic effect (table 2). For most of the tested bacteria, the results of the checkerboard assay were indifferent. A combination of SKEO with gentamicin or norfloxacin did not show any synergistic effect against PAO1 strain. SKEO combined with norfloxacin showed a maximum effect against PA11 (FICI: 0.37). While SKEO reduced the MIC value of gentamicin and norfloxacin by up to 4- and 8-fold, respectively.

Effect of SKEO on the Expression of *mexE*, and *mexY* Genes

Gel electrophoresis of RT-PCR products confirmed the specificity of RNA primers. Subsequently, the qPCR analysis was conducted to reveal the quantitative gene expression of *mexE*, *mexY*, and *gyrA*. Based on the melting curves of the amplified genes, there were no non-specific amplicons or primer dimers.

The one-way ANOVA test showed a significant reduction in the expression of *mexE* and *mexY* genes in the presence of SKEO for all isolates (P<0.001). The Livak method also revealed that different concentrations of SKEO significantly reduced *mexE* expression (9.7-fold) and *mexY* (4.99-fold) compared with the control gene. As shown in table 3, pairwise comparisons of the groups using the *post hoc Tukey HSD test* showed that the efficacy of SKEO on the gene

expression of *mexE* was about two times more than the *mexY* gene. Moreover, the expression of *mexE* gene was 2.18-fold greater than *mexY* gene before treatment.

Discussion

In the present study, the MIC values of SKEO were in the range of 6 to 12 µg/mL, and PAO1 was the most sensitive strain. Treating the MDR strains of *P. aeruginosa* to SKEO combined with antibiotics showed a FICI value between 0.37 and 0.45, indicating a synergistic effect. SKEO in combination with gentamicin or norfloxacin did not show any synergistic effect against PAO1 strain. SKEO combined with norfloxacin showed a maximum effect against PA11 (FICI: 0.37). While SKEO reduced the MIC value of gentamicin and norfloxacin by up to 4- and 8-fold, respectively. There was a significant reduction in the expression of *mexE* and *mexY* genes in the presence of SKEO for all isolates (P<0.01). Different concentrations of SKEO significantly reduced *mexE* expression (9.7-fold), and *mexY* (4.99-fold) compared with the control gene. Moreover, the efficacy of SKEO on the gene expression of *mexE* was about two times more than the *mexY* gene. Additionally, the expression of *mexE* gene was 2.18-fold greater than *mexY* gene before treatment.

The majority of developed chemical EPIs have many harmful effects. Hence, the use of natural

EPIs such as plant extracts is recommended. It is known that phytochemicals can damage bacterial cell walls, resulting in the permeability of the cell membrane.¹⁹ These membrane-damaging compounds are stress factors and can subsequently activate efflux pumps gene expression.²⁰

MexEF-OprN confers resistance to doripenem and meropenem, but this resistance could be due to the low expression of OrpD porin.⁴ Aminoglycosides act as poor substrates for efflux pumps due to their hydrophilic nature. Studies have shown that they are transported by a large number of efflux pumps (MexXY-OprM) from the resistance-nodulation-division superfamily.³ We tested the effect of SKEO on the expression of MexEF-OprN and MexXY-OprM pumps to assess the role of efflux pumps involved in antibiotic resistance. We found that SKEO reduced the expression of *mexEF-OprN* and *mexXY-OprM* genes. Moreover, SKEO combined with gentamicin or norfloxacin resulted in a decrease in their MIC values.

As expected, an acceptable convergence was observed between checkerboard data and the expression of *mex* genes in all clinical isolates, except for two strains that FICI was not determined. The results showed that SKEO inhibited bacterial growth at a lower dose than gentamicin and norfloxacin. Therefore, SKEO can be more useful than antibiotic therapy due to its mild side effects. In line with our findings, Bahador and colleagues showed that in the presence of SKEO, the expression level of the *bap* gene in *Acinetobacter baumannii* decreased by 24-fold.²¹ Other studies have also reported that SKEO could reduce the expression of *mexR*, *mexA*, and *exoS* genes in *P. aeruginosa* strains.^{17, 22} In our earlier study, we reported that SKEO had a significant effect on reducing the expression of *lasA* and *lasB* of *P. aeruginosa*.²³ Similarly, we showed the inhibitory effect of SKEO on the expression of efflux pumps involved in antibiotic resistance.

In a previous study, the reported MIC value of the acetic extract of *Clinopodium vulgare* was similar to our results, while no effects were observed in the presence of ethanolic extract. The difference can be attributed to the rapid evaporation of ethanol and the presence of *P. aeruginosa* strains with high resistance to gentamicin.¹⁹ Despite a 2- to 4-fold reduction in the MIC value of gentamicin by SKEO, we did not observe any synergistic effect. This meant that the *mexY* gene could not be the only resistance factor against gentamicin. Therefore, further studies are required to identify other possible resistance mechanisms. Finally, it seems that

SKEO can be used as a significant EPI in the treatment of patients infected with MDR strains of *P. aeruginosa*. However, it is suggested to assess its pharmacokinetics, potency, and toxicity in human cells. The main limitation of the present study was a low number of isolates, which should be addressed in future studies.

Conclusion

S. khuzistanica essential oil reduced the expression of efflux pumps and the MIC values of norfloxacin and gentamicin *in vitro*.

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Conflict of Interests: None declared.

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