

The Study of *Satureja khuzestanica* Essence Inhibitory Effects against Housekeeping Gene DNA Gyrase A and T3ss and T2ss of *Pseudomonas aeruginosa* with RT-PCR Technique

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Pseudomonas aeruginosa is an opportunistic pathogen that can cause severe hospital-acquired infections, especially in immunocompromised hosts. *Paeruginosa* is important for its resistance to antibiotics. Pathogenic microorganisms have to face hostile environments while colonizing and infecting their hosts. Unfortunately, they can cope with it and have evolved a number of complex secretion systems, which direct virulence factors either at the bacterial cell surface into the environmental extra cellular milieu or into the host cell cytosol. The Gram-negative opportunistic human pathogen *Pseudomonas aeruginosa* possesses abroad panel of secretion systems. Five of the six secretion machines characterized in Gram-negative bacteria are at *P. aeruginosa* disposal, sometimes in several copies. All these machines are dedicated to the specific secretion of exoproteins, which display various activities useful for bacterial adaptation to the environment or for bacterial pathogenicity. In this study inhibitory effects of *Satureja khuzestanica* extract, an endemic plant of Iran, on the expression level of T2SS, and T3SS genes in *P. aeruginosa* were investigated. For this purpose, MIC was determined for *P. aeruginosa*. Then, bacteria were treated with *S. khuzistanica* extract. T2SS, T3SS and gyrA genes expression in treated and non-treated bacteria, before and after treatment was evaluated using RT-PCR technique. Surprisingly, the expression level of T2SS and T3SS genes was decreased in the presence of *S. Khuzestanica*. However, the expression of gyrA gene that was used as an internal control was not altered before and after treatment with this herb. Based on the results; *S. Khuzestanica* could play a major role in lowering the *P. aeruginosa* resistance to drugs, by reducing T2SS, T3SS genes expression. According to results of current research we hope in future be used it to the clinic with a wider range as a complementary therapy and also for surgery operation or ointment.

Key words: RT-PCR, *Satureja khuzestanica*, *Pseudomonas aeruginosa*, MIC, Carvacrol.

Pseudomonas aeruginosa is a ubiquitous Gram negative pathogen widespread throughout the environment that it leads to cause of opportunistic infections in humans¹. In normal hosts, with an intact epithelial barrier, *P. aeruginosa* rarely causes disease. However, in the setting of

epithelial damage, as is seen in immunocompromised and hospitalized patients, *P. aeruginosa* is a common cause of nosocomial infections. Most of these are acute infections, including sepsis, ventilator-associated pneumonia, and infections in post operative wound and burn patients. *P. aeruginosa* also, chronically colonizes Cystic Fibrosis (CF) patients leading to severe pulmonary damage and death. Despite treatment with appropriate antibiotics, mortality remains as

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high as 40% in acute infections and multi-drug resistant isolates are increasingly reported. *P. aeruginosa* has a large number of secreted, virulence factors that rely on specialized export systems including the type I, II, III, V, and the recently discovered type VI secretion systems²⁻³.

In *P. aeruginosa*, the Xcp(extracellular protein) T2SS is encoded by a set of 11 genes organized in two divergent operons, xcpP to Q and xcpR to Z, whereas a 12th gene, xcpA/pilD, is located out-side of the 2xcp operons⁴. This interaction between the GspC(XcpP) and the GspD(XcpQ) components seems to be crucial and confers specificity to the secretion system⁵. Interestingly, two other genes, xphA and xqhA, homologous to xcpP and xcpQ, respectively, have been identified⁶.

One could note that secretins are found in two different secretion pathways, the T2SS and the T3SS. Once the needle is assembled, the various exoproteins are transported through this channel. Obviously, this is performed in a kinetic and ordered manner. The translocators PopB, PopD, and PcrV may be the first proteins transported to form a pore within the eukaryotic cell membrane, in the continuity of the PscF needle. PopB and PopD have hydrophobic domains, which allow their insertion into the eukaryotic cell membranes. PcrV is localized at the tip of the needle, where this scaffold protein may facilitate the assembly of the translocation pore⁷.

Satureja khuzestanica, from Lamiaceae family is an Iranian endemic plant, famous for its medical uses as an analgesic and antiseptic in folk medicine⁸. It is mostly found in western and southern part of Iran⁹. Recently, antiviral, antibacterial, antifungal, and antiprotozoal effects were investigated from various species of *Satureja*⁹⁻¹⁰. However, the possible effect of *Satureja khuzestanica* on decreasing the resistance of *P. aerogenase* against antibiotics and the mechanisms involved have not yet been studied. The antibacterial activity of the *S. khuzistanica*' soil might be due to main phenolic components, Carvacrol and Thymol¹¹. Carvacrol is also found in Thyme, However, its high ratio in *S. khuzistanica* has discriminated this plant from other herbs with antimicrobial effects¹²⁻¹³. In view of this and with regard to the antimicrobial effect of *S. khuzistanica* against *P. aerogenase* and also resistance of this strain to variety of antibiotics, this study aimed to

test this hypothesis that *S. khuzistanica* extract may alter the expression of T2SS, T3SS genes, and thus may lead to a lower susceptibility of this strain to antibiotics.

MATERIALS AND METHODS

Plant extraction procedure

S. khuzistanica were collected in Khoramabad, Iran in 2013. Essential oil was prepared by steam distillation of the aerial parts of the plant. Oil after drying with sodium sulfate was kept at 4 °C until use in GC injection system. For this study; the microbial strains were collected from Baqiyatallah hospital. Susceptibility testing of antibiotics were performed. The resistant strains were subjected to *S. khuzistanica* extract. To evaluate the antimicrobial effects of *S. khuzistanica* essential oil, diffusion method (disk diffusion) was used according CLSI 2013. Dimethylsulfoxide (DMSO) was used to dissolve the essential oil and then diluted to the concentration 0.1 µl/ml. Culture carried out by a sterile swab and the resulting suspension was cultured for 24 h and then inoculated onto Mueller Hinton agar blank discs (Merck, Germany) with a diameter of 6 mm; containing 30 µl of the essential oil was placed on Muller Hinton agar medium. After 24 h of incubation at 37 °C, zones of growth inhibition were measured. The experiment was repeated 3 times. Disks containing 30 µl of dimethyl sulfoxide were used as a negative control. Determination of MIC carried out as microdilution according to CLSI. The standard antibiotic discs of Furazolidone (100mcg/disc), Erythromycin (15mcg/disc), PolymyxinB (30mcg/disc), Ceftazidime (30mcg/disc) were prepared to evaluate the antimicrobial susceptibility from padanteb, Tehran, Iran. Specific primers for T2SS, T3SS and gyrA genes (Table 1) were designed using Genscript software (GenScript Real-time PCR (TaqMan) Primer Design). After determination of MIC for each strain, the strain of interest was subjected to the determined MIC concentration. Then, the RNA was isolated from bacteria exposed to the herbal extract (cases) and those lacking *S. khuzistanica* in their media (controls) according to the manufacturer's protocol (Cinnagen). For both samples, cDNA was synthesized and the alterations in the expression level of T2SS, T3SS and gyrA genes were identified

by RT-PCR method (Cinnagen) with the following conditions: 3 minutes at 95°C (1 cycle), 30 seconds at 95 °C (35 cycles), 30 seconds at 54 °C (35 cycles), 1 minute at 72 °C (35 cycles) and 10 minutes at 72°C for final extension. A housekeeping gene, *gyrA*, was used as an internal control.

RESULTS AND DISCUSSION

The essential oil of *S. khuzistanica* was active against *Paerogenase* in with range from MIC=0.31µg/ml which remarkably was exhibited higher activity relative to the referent antibiotics.

In this study, antimicrobial susceptibility of *Pseudomonas aeruginosa* to different antibiotics was determined and results as mean inhibition zone for a variety of antibiotics are given in Table 2.

The results of RT-PCR before and after bacteria treatment revealed that the expression of T2SS and T3SS genes were remarkably reduced in the presence of *S. khuzistanica* extract (Figure1), while these two genes were highly expressed before the exposure of bacteria with this herb.

As expected, expression of *gyrA* gene was relatively constant in samples and controls. Expression of the *gyrA* gene served as an internal control to ensure that equal amounts of RNA were used in all RT-PCRs.

Pseudomonas aeruginosa treatment is

difficult with existing antibiotics, but may in addition develop resistance after unsuccessful treatment. Thus, it is considered as an increasing threat to the community. The intrinsic antibiotic resistance of *Pseudomonas aeruginosa* may be associated with the limited permeability of bacteria's outer membrane¹⁴. The use of medicinal and herbal plant to treat infectious diseases is common in many countries¹⁵. *Satureja khuzestanica* has been used as a medicinal herb since the ancient times. Carvacrol is one of the major compounds in this plant, which is easily dissolved in ethanol. Moreover, antioxidant and antibacterial properties of this plant could be attributed to the presence of this agent¹⁶. Numerous studies have been published on *S. khuzistanica* extract¹⁶⁻¹⁷. Amanlou *et al.* used *S.khuzestanica* extract for treatment of mild aphthousulcers¹⁶. Antifungal¹⁸ and antimicrobial¹⁹ effects of *S. khuzistanica* leaf extract had been also demonstrated. In a study carried out by Amiri *et al.* the impact of *S. khuzistanica* extract on some bacteria, causing hospital infections, was investigated. They showed a strong inhibitory effect for this plant against common nosocomial bacteria²⁰.

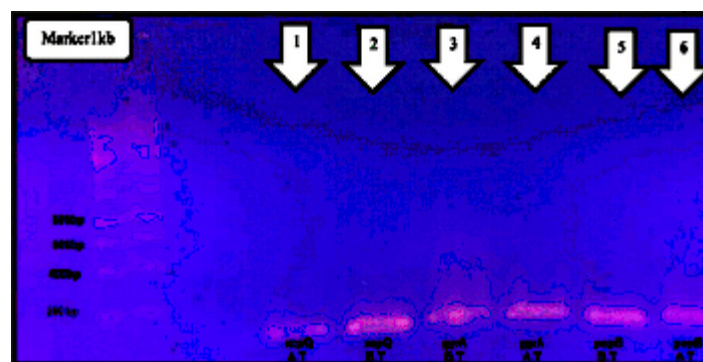
In a study carried out by Kim *et al* in 1995, anti-bacterial effects and MIC and MBC measurement of carvacrol on *Salmonella typhimurium* and strain resistant to its Rifampicin

Table 1. The list of Primers used in RT-PCR and their sequences

Primer	Sequence	size
PrimerF popB	GGATCAGTGCAATAGCTTCG	120bp
PrimerR popB	GTACCGACTGGGAGACCACT	120bp
PrimerF xcp Q	CAGAAAGCGGAGATTCTGGT	70bp
PrimerR xcp Q	CTTCGCTGTTGGTGGTGTAG	70bp
PrimerF gyrA	GGTCTGGGCATAGAGGTTGT	121bp
PrimerR gyrA	GAAGATCGAGGGTATTTCCG	121bp

Table 2. Mean inhibition zone of clinical strain of *Pseudomonas aeruginosa* against various antibiotics (Mean± SE.)

Antibiotic	Mean inhibition zone(mm)±SE	Antibiotic	Mean inhibition zone(mm) ±SE
Furazolidone	0.00 ± 0.00	Ceftriaxone	17.66 ± 0.33
Erythromycin	10.66 ± 0.66	Gentamicin	13.33 ± 0.88
Ceftazidime	6.33 ± 3.17	Ampicillin	13.00 ± 0.57
Polymyxin B	11.66 ± 0.88	Imipenem	22.66 ± 0.33



Line1 &2 are related to *xcpQ* after and before treatment with *S. khuzestanica* consequently. Line3&4 related to house keeping gene *gyrA* before & after with *S. khuzestanica* consequently. Line5&6 are related to *popB* after and before treatment with *S. khuzestanica* consequently.

Fig. 1. Results of RT PCR for genes T2ss and T3ss

in Tryptic Soy agar medium (using paper disks smeared with given concentrations of carvacrol and defining the zone to prevent growth) and in Tryptic Soy agar by measuring growth turbidity using spectrophotometer at a wave length of 540nm then cultured on Tryptic Soy agar were examined. They showed that carvacrol had strong antibacterial effects on two studied Strain with MIC:250µg/ml. Our research suggested that carvacrol has antimicrobial properties and its mean controlling concentration on *Pseudomonas aeruginosa* was MIC: 0.31µg/ml also it showed that this research was consistent with other research²¹.

Eunkyoung Kim study showed that carvacrol had liver anti-fat property in highly-fed mice. This research suggested that carvacrol can reduce gene expression of LXR±, Leptin, FAS, and SREBP1c and on the other hand it can increase SIRT1 gene expression. These genes are involved in fatty acids metabolism and the results of this study show that carvacrol regulates expression of genes involved in lipids metabolism. Our study's results also indicate that *Satureja khuzestanica* Essence, especially its carvacrol are involved in expression of antibiotic-resistant genes in *Pseudomonas aeruginosa*, reducing its expression to a great extent. In the present study, we showed that *Satureja khuzestanica* Essence is able to control antibiotic-resistant genes in prokaryotes²².

Inhibition significantly decreased MICs for both antibiotic-susceptible and resistant bacteria, and resulted in a decreased frequency of

mutant *P. aeruginosa* bacteria that were highly resistant to fluoroquinolones. In accordance with this study, our results also showed that inhibiting the T2SS, T3SS by *S. khuzestanica* caused a decreased level of MICs for resistant *P. aeruginosa* bacteria. In the current study, RT- Technique was applied because it is a rapid and highly applicable technique for evaluating the expression profile of the target gene(s) and provides qualitative or semi quantitative information of mRNA levels. However, further studies are required to quantify the expression of the studied genes and identifying similar medicinal herbs that can block secretory system and thus extend the life of existing antibacterial drugs could be beneficial. In summary, our data suggest that medicinal plant extracts, particularly of *Satureja khuzestanica*, may provide suitable compounds for clinical utility as inhibitors of secretory system for *P. aeruginosa* strain. According to results of current research we hope in future be used it to the clinic with a wider range as a complementary therapy. Additional clinical research and trials are necessary to completely confirm the above results for medical purposes. Thus it can be deduced the natural products have antimicrobial power higher even than synthetic and semi-synthetic antibiotics.

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