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The Study of Inhibitory Effects of *Satureja khuzestanica* Essence against Exos Gene of MDR *Pseudomonas aeruginosa* by RT-PCR Technique

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that can cause severe hospital-acquired infections, especially in immune-compromised hosts. *P. aeruginosa* is notorious for its resistance to antibiotics. Exoenzyme S is one of the several toxins involved in Spreading of infections in burns. It's been revealed that deletion of genes encoding the components of exoS, in wild-type *P. aeruginosa*, confers hyper susceptibility to anti phagocytic agents. Antimicrobial and antifungal properties of some herbal medicines were numerously reported. In this study the effect of *Satureja khuzestanica* extract, an endemic plant of Iran, on the expression level of exoS gene in *P. aeruginosa* were investigated. For this purpose, MIC was determined for *P. aeruginosa*. Then, bacteria were treated with *S. khuzistanica* extract. ExoS and gyrA genes expression in treated and non-treated bacteria, before and after expose was evaluated using RT-PCR technique.

Surprisingly, the expression level of exoS gene 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 exoS gene 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 and etc.

Keywords: RT-PCR; *Satureja khuzestanica*; ExoS; MDR; Gene expression inhibition

Inroduction

Pseudomonas aeruginosa is a clinically significant opportunistic pathogen and one of the leading causes of nosocomial infections worldwide [1]. This organism has an outer membrane with a low level of permeability and is thereby intrinsically resistant to a wide variety of commonly used antibiotics [2]. Pseudomonas infections are commonly reported in burns, surgery, urinary tract infections (UTI), and pulmonary diseases such as cystic fibrosis (CF) [1]. This diversity of pseudomonas infections is due to the development of various adaptive mechanisms such as the nutritional and metabolic pathways as well as the regulation of gene expression [3].

Pseudomonas aeruginosa is as opportunistic pathogen that primarily infects immune compromised individuals and causes life-threatening infections in burn patients and exacerbates lung problems in individuals with cystic fibrosis. One of major virulence factors is exoenzyme S (ExoS) that it has been proposed to act as an antiphagocytic factor [4], thus it enable the bacteria to evade to host immune system. ExoS was characterized as an ADP-ribosylating enzyme by Iglewski et al. [5], and studies focused this property to activity to its carboxy terminus [6]. Then ExoS was translocated into human epithelial cells by the contact-dependent type III secretion (T3SS) system [7], subsequently ADP-ribosylate (ADPR) targets multiple substrates, including the low-molecular-weight G (LMWG) proteins Ras, RalA, certain Rab proteins, Rac1, and Cdc42 [6,8-25]. ExoS was found to also include a GTPase-activating protein (GAP) activity that targets the LMWG proteins Rho, Rac1, and Cdc42, which affect eukaryotic cell cytoskeletal structure [8]. T3SS-translocated ExoS can exert complex effects on eukaryotic cell function, including inhibition of DNA synthesis, alterations in cell morphology, microvillus effacement, and loss of cellular adherence, in addition to its antiphagocytic or anti-invasive effects [9-12].

Besides, the rapid spread of bacteria expressing multidrug resistance (MDR) has necessitated the discovery of new antibacterial and resistance-modifying agents [13]. Herbal medicine has been long used against microbial and they were confirmed to be safe and efficient with fewer side effects compared to chemical pharmaceuticals. *Satureja khuzestanica*, from Lamiaceae family is an Iranian endemic plant, famous for its medical uses as an analgesic and antiseptic in folk medicine [14]. It is mostly found in western and southern part of Iran [15]. Recently, antiviral, antibacterial, antifungal, and antiprotozoal effects were investigated from various species of Satureja [16-19]. However, the possible effect of *Satureja khuzestanicaon* decreasing the resistance of *P. aeruginosa* against antibiotics and the mechanisms involved have not yet been studies. *Satureja khuzestanica* has been investigated as an antibacterial agentin this research. The antibacterial activity of the *S. khuzestanica* soil might be due to main phenolic components, Carvacrol and Thymol [20]. Carvacrol is also found in Thyme, However, its high ratio in *S. khuzistanica* has discriminated this plant from other herbs with antimicrobial effects. Carvacrol and Thymol are phenolic compounds and probably provides antibacterial activity on cell membrane (oxidative phosphorylation), protein biosynthesis and DNA and RNA. Not only carvacrol decrease reduction of ATP synthesis by a dissipation of the proton motive force but also other (secondary) effects of carvacrol may result in the bactericidal or bacteriostatic action. For example, an inhibition of several enzymes due to leakage of essential ions, loss of turgor pressure, influence on DNA synthesis, reduced metabolic activities, and other processes in the cell can be a cause of the decreased viability during exposure to carvacrol.

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A loss of membrane integrity due to disturbance of hydrophobic interactions between lipids and proteins is often an important factor when considering the activity of toxic compounds [21,22]. Although, the inhibition of the virulence genes could restore the drug activity against the resistant strains and minimizes their further development [23,24]. In view of this and with regard to the antimicrobial effect of *S. khuzestanica* against *P. aeruginosa* and also resistance of this strain to variety of antibiotics, this study aimed to test this hypothesis that S. *khuzestanica* extract may alter the expression of exoS 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. The aerial parts of the *Satureja khuzestanica* plant were collected during the flowering stage in June 2013 from Khoramabad in the Lorestan province of Iran. The plant was identified by the Department of Botany of the Research Institute of Forests and Rangelands (TARI) in Tehran. A voucher specimen (No. 58416) has been deposited at the TARI Herbarium. The plant was cultivated in Khoramabad and the aerial parts of the plant were collected during the flowering stage. The aerial parts were air dried at ambient temperature in the shade and hydro distilled using a Clevenger type apparatus for 5 h, giving yellow oil in a 0.9% yield. The oils were dried over anhydrous sodium sulfate and stored at 4°C (Table1).

For this study, the microbial strains were collected from Motahari hospital in burn units. 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 concentrations (500-0.25 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. In order to determine antibiotic resistance were used antibiotic discs according to CLSI 2013 and zones of growth inhibition were compared to *S. khuzistanica* essential oil. Determination of MIC carried out as micro dilution according to CLSI. The standard antibiotic discs of Furazolidone (100 mcg/disc), Erythromycin (15 mcg/disc), PolymyxinB (30 mcg/disc), Ceftazidime(30 mcg/disc), Ceftriaxone(30 mcg/disc), Gentamicin(10 mcg/disc), Ampicillin(10 mcg/disc) and Imipenem(10 mcg/disc) were prepared to evaluate the antimicrobial susceptibility from padtan teb, Tehran, Iran.

Specific primers for exos genes (Table 2) 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 synthetized and the alterations in the expression level of exoS and gyrAgenes 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 57°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

The essential oil of *S. khuzestanica* was active against *P. aeruginosa* the range from MIC=0.5 µg/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 3.

The results of RT-PCR before and after bacteria treatment revealed that that the expression of exoS gene were remarkably reduced in the presence of *S. khuzestanica* extract (Figure 1), while this gene was 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.

Discussion

Pseudomonas aeruginosa is MDR Gram-negative bacterium therefore difficult to treat 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 membrane [9] or overexpression of resistant genes [25-27]. The presence of virulence

S. No.	%c	Rib	Rta	Compound
1	0/28	935	4/081	α-Pinene
2	0/39	990	5/157	β-Myrcene
3	0/49	1016	5/77	α-Terpinene
4	3/11	1023	5/964	p-Cymene
5	0/19	1027	6/067	β-Phellandrene
6	1/24	1056	6/828	ɣ-Terpinene
7	0/91	1098	7/944	Linalool
8	0/35	1162	9/89	Borneol
9	0/65	1173	2/26	Terpinene-4-o1
10	0/19	1291	13/992	Thymol
11	90/88	1296	14/164	Carvacrol
12	0/15	1413	17/757	(Z)-Caryophyllene
13	0/21	1502	20/464	β-Bisabolene
14	0/18	1574	22/57	Caryophyllene oxide

RTc: Retention Time (min); RIb: Retention Indices Determined on HP-5MS Capillary Column; %a: Calculated from TIC Data.

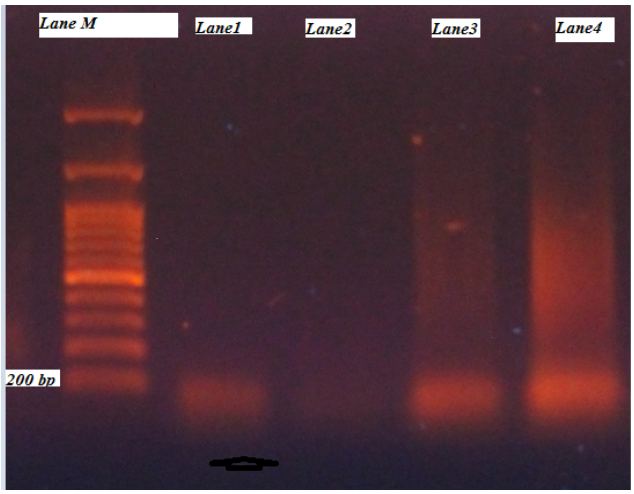
Table1 : Components of *S. khuzestanica* essence with GC mass.

Primer	Sequence	size
Primer F exoS	AGAGAGCGAGGTCAG CAG AG	153 bp
Primer R exoS	ATGCCGGTGTAGAGACCAAG	
Primer F gyrA	GGTCTGGGCATAGAGGTTGT	121 bp
Primer R gyrA	GAAGATCGAGGGTATTTCCG	

Table2: Primer sequences designed exos and gyrA genes.

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

Table 3: Mean inhibition zone of clinical strain of *Pseudomonas aeruginosa* against various antibiotics (Mean ± SE.)



Lane M: Marker DNA 1 kb; Lane 1: exoS gene previous exposure with *S. khuzestanica* essence; Lane 2: exoS after exposure to *S. khuzestanica* essence; Lane 3: gyrA gene previous and Lane4 after exposure to *S. khuzestanica* essence.

Figure1: Results of RT-PCR for exos gene and gyrA.

factors and their broad substrate profile is the cause of intensive infection [28]. It is therefore imperative that new antibiotics, infection-modifying agents and, more specifically, exoS inhibitors are identified [13].The use of medicinal and herbal plant to treat infectious diseases is common in many countries [29]. *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 agent [30]. Numerous studies have been published on *S. khuzestanica* extract [30-33]. A Manlou et al. used *S. khuzestanica* extract for treatment of mild aphthousulcers [30]. Antifungal [34] and antimicrobial [35] effects of *S. khuzestanica* leaf extract had been also demonstrated. In a study carried out by Amiri et al. the impact of *S. khuzestanica* extract on some bacteria, causing hospital infections, was investigated. They showed a strong inhibitory effect for this plant against common nosocomial bacteria [31]. Also, a separate study revealed that the extracts from plants that are used as herbal medicinal products contain inhibitors of efflux pump in Gram-negative bacteria [36]. However, the influence of *S. khuzestanica* on the expression of exoS gene has not yet been investigated and this is the first study reporting the inhibitory effect of this plant on it. Based on the results obtained in this study, the extract of *S. khuzestanica* had an impending role on exoS by reducing the expression of exoS. Lomovskaya et al. had formerly shown the consequences of inhibiting the efflux pumps of *P. aeruginosa* by a genetic approach [37, 38]. In accordance with this study, our results also showed that inhibiting the exoS by *S. khuzestanica* caused a decreased level of MICs for resistant *P. aeruginosa* bacteria. The results of research indicate that in compare to negative control and antibiotics *S. khuzestanica* essence enable decrease MIC level. In the current study, RT-PCR technique was applied because it is a rapid and highly applicable 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 virulence genes 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 virulence genes for *P. aeruginosa* strain. According to results and due to the high resistance to more drugs and disinfectants in *P. aeruginosa* and high prevalence of nosocomial infections and enormous economic costs and the restrictions on the use of broad-spectrum drugs in persons with Immuno-compromised applications of native compounds against these pathogens resulted in these which can be effective enough to reduce the rate of infection transmission. 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. This study showed the emergence of drug resistance in *P. aeruginosa* against the antimicrobial agents being routinely used for treatment and revealed the likely presence of co-selected traits that result in highly virulent in resistant strains. Further clinical investigations are warranted to combat infections caused by this important human pathogen by inhibition of important virulence factors. The data demonstrate that carvacrol is very potent inhibitor of exoenzyme S. In this study, expression of mRNA by RT-PCR was qualitative analysis; we recommend quantitative real-time RT-PCR analysis and transcriptome are preferred for a gene expression.

Authors' Contributions

All authors had equal role in design, work and manuscript writing.

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