THE STUDY OF INHIBITORY EFFECTS OF SATUREJA KHUZESTANICA ESSENCE AGAINST MEXA AND MEXR EFFLUX GENES OFPSEUDOMONAS AERUGINOSA BY RT-PCR

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ABSTRACT

Background: Pseudomonas aeruginosa is an opportunistic pathogen that can cause severe hospital-acquired infections, especially in immunocompromised hosts. P.aeruginosa for its resistance to antibiotics. Efflux pump is one of the several mechanisms involved in intrinsic resistance of these bacteria to antibiotics. It has been revealed that deletion of genes encoding the components of MexAB–OprM of efflux system, in wild-type P. aeruginosa, confers hypersusceptibility to a variety of antimicrobial agents. Antimicrobial and antifungal properties of some herbal medicines were reported. Objectives: In this study the effect of Saturejakhuzestanica extract, an endemic plant of Iran, on the expression level of mexA, and mexR genes in P. aeruginosa were investigated. Materials and Methods: In this study, MIC was determined for P. aeruginosa. Then, bacteria were treated with S. khuzistanica extract. MexA, mexR and gyrA genes expression in treated and non-treated bacteria, before and after treatment was evaluated using RT-PCR technique. Results: Surprisingly, the expression level of mexA and mexR genes was decreased in the presence of S. Khuzestanica. However, the expression of gyrAgene 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 mexA genes expression. Conclusions: 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.

Keywords: RT-PCR, Saturejakhuzestanica, MexAB–OprM, Efflux pump, Gene expression inhibition.

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1. INTRODUCTION

Pseudomonas aeruginosa is a clinically significant opportunistic pathogen and one of the leading causes of nosocomial infections worldwide (Askoura *et al.*, 2011). 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 (Angus, 1982). Pseudomonas infections are commonly reported in burns,surgery, urinary tract infections (UTI), and pulmonary diseases such as cystic fibrosis (CF) (Askoura *et al.*, 2011). 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 (Leid, 2009).Of these resistance mechanisms in *Pseudomonas aeruginosa*, the active efflux pumps play a crucial role in extruding the antibiotics outside the bacterial cells (Askoura *et al.*, 2011).

The *P. aeruginosa* genome carries several xenobiotic efflux pumps, including MexAB-OprM that is a member of RND (resistance nodulation division)family of exporters (Saier, 1994). OprM, is the product of the third gene of a multidrug resistance operon, mexA-mexB-oprKwhich has been renamed as mexA-mexB-oprM (Gotoh, 1995). MexB, OprM, and MexA (Nikaido, 1994; Nakae *et al.*, 1997; Poole, 2001) subunits function as the substrate recognizing energy-transmitting subunit (Guan, 1999; Eda *et al.*, 2003) the adapter protein connecting MexB and OprM (Dinh *et al.*, 1994; Akama, 2004; Higgins, 2004), and the antibiotics discharge duct protein (Nakae *et al.*, 1997; Poole, 2001; Akama, 2004), respectively.Overproduction of MexAB-OprM was shown to be associated with the intrinsic resistance in clinical isolates of *P. aeruginosa*. Numerous reports had previously suggested that overexpression of MexAB-OprM system innalB multidrug-resistant mutants may be due to mutations in mexRgene (Poole, 1996; Srikumar *et al.*, 1997; Ziha-Zarifi, 1999). The mexR gene is transcribed divergently from the mexAB-oprM genes and the resulting protein is a repressor from MarR family of regulators (Poole, 1996). MexR binds to the mexR-mexAintergenic region, overlapping promoters for mexR and mexAB-oprM (Evans *et al.*, 2001), and may contribute to repression of these promoters (Poole, 1996; Srikumar *et al.*, 1997).

Inhibiting the efflux pumps could be beneficial as it may improve the efficiency of various antibiotics against resistant bacteria (Lomovskaya and Watkins, 2001). Besides, the rapid spread of bacteria expressing multidrug resistance (MDR) has necessitated the discovery of new antibacterial and resistance-modifying agents (Stavri *et al.*, 2007). 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. *Saturejakhuzestanica*, from Lamiaceae family is an Iranian endemic plant, famous for its medical uses as an analgesic and antiseptic in folk medicine (Scully *et al.*, 2003). It is mostly found in western and southern part of Iran (Van Baren, 2006). Recently, antiviral, antibacterial, antifungal, and antiprotozoal effects were investigated from various species of Satureja (Sahin, 2003; Skocibusic and Bezic, 2004; Sonboli, 2004; Tampieri, 2005; Van Baren, 2006). However, the possible effect of *Satureja khuzestanica*on decreasing the resistance of

P.aeruginosa against antibiotics and the mechanisms involved have not yet been studied. The antibacterial activity of the *S. khuzestanica's*oil might be due to main phenolic components, Carvacrol and Thymol (Deans and Svoboda, 1989).

Carvacrol is also found in Thyme, however, its high ratio in *S. khuzistanica* has discriminated this plant from other herbs with antimicrobial effects (Dorman and Deans, 2000; Lambert, 2001). Altogether, the inhibition of the efflux pumps could restore the drug activity against the resistant strains and minimizes their further development (Mahamoud, 2007; Zhang and Mah, 2008). 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 mexA, mex R genes of the RND efflux pump, MexAB-OprM, and thus may lead to a lower susceptibility of this strain to antibiotics.

2. MATERIALS AND METHODS

2.1. Plant Extraction Procedure:

S. khuzistanica were collected in Khoramabad, Iran in 2013 Iran. 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 Baqiyatallahhospital surgery units, ICU and burns ward. Susceptibility testing of antibiotics neomycin, gentamicin, amikacin, kanamycin, and oxacillin 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 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 37⁰C, 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 (100μg/disc), Erythromycin (15μg/disc), PolymyxinB (30μg/disc), Ceftazidime(30μg/disc), Ceftriaxone(30μg/disc), Gentamicin(10μg/disc), Ampicillin(10μg/disc) and Imipenem(10μg/disc) were prepared to evaluate the antimicrobial susceptibility from padtanteb, Tehran, Iran.

Specific primers for mexA, mexR 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 synthetized and the alterations in the expression level of mexA, mexR 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.

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

Primer	Sequence	size
PrimerFmexA	AACAGCTCGACCCGATCTAC	146bp
Primer R mexA	GTATTGGCTACCGTCCTCCA	
Primer F mexR	AGGTTTCTTCCCTCCAGCTC	126bp
Primer R mexR	CGACGTCCATGTATTGAAGC	
Primer F gyrA	GGTCTGGGCATAGAGGTTGT	121bp
Primer R gyrA	GAAGATCGAGGGTATTTCCG	

3. RESULTS

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

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

The results of RT-PCR before and after bacteria treatment revealed that that the expression of mexA and mexR genes were remarkably reduced in the presence of *S. khuzestanica* extract (Figure 1), 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.
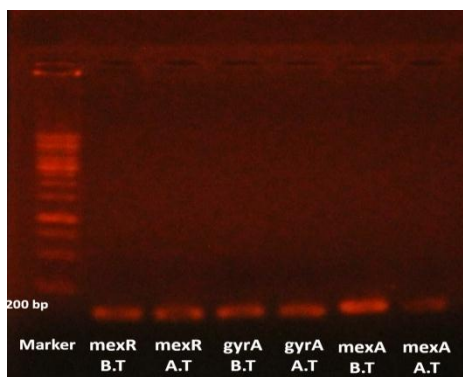
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Figure-1. RT- PCR result showed that the expression of mexR and mexA genes was decreased after treatment with *S. khuzestanica*, whereas, the expression of gyrA gene had remained constant throughout the study, B.T: before treatment, A.T: after treatment.

4. DISCUSSION

Pseudomonas like other Gram-negative bacteria is per se 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 (Nikaido, 1994) over expression of efflux pumps, such as MexAB-OprM (Poole, 1993; Poole, 2004; Li and Nikaido, 2009). The presence of these pumps and their broad substrate profile is the cause of the innate resistance to antibiotics in Gram-positive and some Gram-negative bacteria (Piddock, 2006). It is therefore imperative that new antibiotics, resistance-modifying agents and, more specifically, efflux pump inhibitors (EPIs) are identified (Stavri *et al.*, 2007).The use of medicinal and herbal plant to treat infectious diseases is common in many countries (Ankli, 2002). *Saturejakhuzestanica* 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 (Amanlou, 2007). Numerous studies have been published on *S. khuzestanica* extract (Amanlou, 2007; Safarnavadeh and Rastegarpanah, 2011; Zibaei *et al.*, 2012; Amiri, 2013). Amanlou et al. used *S. khuzestanica* extract for treatment of mild aphthous ulcers (Amanlou, 2007). Antifungal (Abbasi, 2014) and antimicrobial (Garvey, 2011) effects of *S. khuzestanica* leaf extracthad 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 (Amiri, 2013). 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 (Garvey, 2011). However, the influence of *S. khuzestanica* on the expression of efflux pump has not yet been investigated and this

is the first study reporting the inhibitory effect of this plant on efflux system. Based on the results obtained in this study, the extract of *S. khuzestanica* had an impeding role on mexAB-OprM by reducing the expression of mexA and mexR genes and therefore caused an efficient function of antibiotics on *P. aeruginosa* bacteria. Lomovskaya *et al.* had formerly shown the consequences of inhibiting the efflux pumps of *P. aeruginosa by a genetic approach* (Lomovskaya and Watkins, 2001). 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 mexAB-OprM efflux pump 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 efflux and thus extend the life of existing antibacterial drugs could be beneficial.

In summary, our data suggest that medicinal plant extracts, particularly of *Saturejakhuzestanica*, may provide suitable compounds for clinical utility as inhibitors of efflux for *P. aeruginosa* strain. According to results and due to the high resistance to more drugs and disinfectants in *A. baumannii* and high prevalence of nosocomial infections and enormous economic costs and the restrictions on the use of broad-spectrum drugs in persons with immunocompromised 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.

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