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STUDY OF INHIBITION OF APHA6 GENE IN *ACINETOBACTER BAUMANII* BY *SATUREJA* AND *THYME* ESSENCE WITH RT-PCR TECHNIQUE

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ABSTRACT

Increasing isolation of multidrug-resistant Acinetobacter baumannii (MDR) has been reported in worldwide and it is recently one of the most difficult nosocomial acquired gram-negative pathogens to control and treatment.These bacteria are one of ESBL producing that great potential have for the rapid development of antibiotic resistance. The indecorous use of antibiotics is often associated with adverse effects on the human health.Because of Satureja and Thyme essence has antimicrobial properties so they can be used against infections caused by MDR Acinetobacter baumannii. With attention to increase in population and tendency to lesser use of synthetic drugs, many of these of medicinal herbs have been replaced. The present study was aimed to investigate the inhibitory effects of Satureja khuzestaniea and Thyme essence against the expression of antibiotic resistance genes aphA-6 and compare to Housekeeping DNA gyraseA in strains of Acinetobacter baumannii with RT-PCR Technique. The major components of Satureja khuzestaniea essence were carvacrol (90.88%), ρ-cymene (3.11%), ϒ terpinene (1.24%), linalool (0.91%) and Thyme essence were thymol (28.8%) and carvacrol (23.46%). Satureja khuzestaniea with MIC (0.3 µl / ml) and Thyme with MIC (0.45µl / ml) has the effect of reducing the expression of antibiotic resistance genes aphA-6 but these are essence no inhibitory effect on the housekeeping DNA Gyrase-A gene. This study indicated that these are essence have inhibitory effects against gene expression of antibiotic resistance aphA-6 with high MIC. Because these are essence has a good inhibitory effect against MDR Acinetobacter baumanii and, aphA-6 gene expression so it can be used as therapeutic complementary no side effects against this bacterium.

Keywords: *Acinetobacter baumannii*, Multidrug-resistant, *Satureja khuzestaniea*, *Thyme*, AphA-6, RT-PCR.

Contribution/ Originality

The paper's primary contribution is finding that *Thyme essence* has exciting properties for gene silence and can introduce it as complementary treatment.

1. INTRODUCTION

Acinetobacter baumannii is a Gram-negative non-fermentative coccobacillus belonging to the family Moraxellaceae that recently it has gained increasing attention as a nosocomial pathogen (Hanlon, 2005). These organisms have been implicated in a diverse range of infections (respiratory tract, bloodstream, skin and soft tissue, prosthetic devices) and are a particular problem in intensive care units where numerous outbreaks have been extremely difficult to control. The rapid emergence and global dissemination of *A. baumanniias* as major nosocomial pathogen is remarkable and demonstrates its successful adaptation to the 21st century hospital environment. (Ku *et al*., 2000). *A. baumannii* is often resistant to a wide variety of antimicrobial agents. Over the last 10 years, A. baumannii emerged as one of the most problematic pathogens as treatment has been limited to only a few antibiotics. The ability of *A. baumannii* to survive for extended periods on environmental surfaces are notorious and are likely important for transmission within the health care setting (Peleg *et al*., 2008). *Acinetobacter* infections are difficult to cure, since strains are often resistant to multiple antimicrobial agents. β-Lactams, quinolones, and aminoglycosides are the major groups of antibiotics used in therapy of infections due to this bacterial genus. *Acinetobacter spp*. Are naturally resistant to cephalosporins (Morohoshi and Salto, 1977) and to low levels of trimethoprim, and they can acquire plasmids or transposons or both which account for antibiotic resistance. Until recently, amikacin remained the most active aminoglycoside in the treatment of infections caused by *Acinetobacter spp*., although it was inactivated in certain strains by a chromosomal 6'-aminoglycoside-acetylating enzyme. However, since 1984, outbreaks of infections due to Acinetobacter spp. resistant to amikacin have been observed in France (Murray and Moellering, 1980). Health problems were caused by *Acinetobacter spp* and possible transition between living and non-living things and as well as long-term survival in the hospital environment enhance the appearance of the bacteria in the hospital environment and infection due to increasing (Gordon and Wareham, 2010). Use of medicinal herbaceous drugs recommended for treatment since ancient periods. Humans have been used and realize their beneficial effects. With the increase in population and urban growth, and reduced use of synthetic drugs, many of these of medicinal herbs have been replaced. (Sharififar *et al*., 2007). The utilization problems of synthetic drugs such as the increasing of antibiotic widespread resistance emrge among microorganisms and economical detriments was induced people tend to herbal treatments.

2. MATERIALS AND METHODS

2.1. Plant Material

Aerial parts of *Satureja khuzestaniea* and Thyme were collected from Barij Essence research farm in May 2010. The voucher specimen was prepared and deposited at the Herbarium of Agriculture Department Research Center of Barij Essence, Kashan, and Iran.

2.2. Extraction, Isolation and Identification of the Oil

The plants were grinded into small pieces (100 g) and subjected to hydro distillation for 6 h using a Clevenger type apparatus. The oil was obtained and dried using anhydrous sodium sulfate and stored in tightly closed dark vial. The oil analysis was carried out using GC and GC/MS. The GC apparatus was an Agilent technology (HP) 6890 system, with HP-5MS capillary column (60 m .0.25 mm i.d., film thickness 0.25mm). The oven temperature program was initiated at 401C, held for 1 min, then increased to 2301C at a rate of 31C/min and held for 10 min. Helium was used as the carrier gas at a flow rate 1.0 ml/min. The detector and injector temperatures were 250 and 2301C, respectively. GC/MS analysis was conducted on a HP 6890 GC system coupled with 5973 network mass selective detector with a capillary column the same as above, carrier gas helium with flow rate 1 ml/min with a split ratio equal to 1/50, injector and oven temperature programmed was identical to GC.

The compounds of the oil were identified by comparison of their retention indices (RI) and mass spectra fragmentation with those on the stored Wiley 7n.1 mass computer library, and NIST (National Institute of Standards and Technology) (National Committee for Clinical Laboratory Standards, 2002).

2.3. Microbial Strains

The number of five Multidrug-resistant strains of *Acinetobacter baumannii* among seventy five strains *Acinetobacter baumannii* from Tehran hospitals that contain of aphA6and housekeeping DNA Gyrase-A gene selected and with PCR technique were approved for this research. *Acinetobacter baumannii* ATCC 19606 was employed in this study as a model reference strain. *A. baumannii* were isolated from clinical specimens such as blood, respiratory secretions, pus and wound swab, cerebrospinal fluid, and urine.

2.4. Antimicrobial Susceptibility Test

Susceptibility to various classes of antibiotics was determined by the disc diffusion method in accordance with Clinical Laboratory Standard Institute 2013 (CLSI) guidelines (Gordon and Wareham, 2010). The testing antibiotics were performed white Amikacin (30 µg), Oxacillin (30µg), Kanamaycin (30µg), Gentamaicin(10µg), Neomycin (30µg) and Imipenem (10 µg). The antimicrobial activity of the essential oil and its main component was determined by the disk diffusion method (National Committee for Clinical Laboratory Standards, 2002).Use (DMSO) dimethyl sulfoxide for dissolve essential oil for this to work 10 µl from essence was dissolved in 90 µl DMSO.The final concentration on about 0.1 (mg/ml). Briefly, 0.1 ml of microorganism (1.5×10^{8} CFU /ml) was spread on Mueller-Hinton agar plates .Afterward Sterile 6 mm disks, containing 3 µl (concentration of about 0.1 (mg/ml))of essential oil were placed on the microbial lawns. These plates were incubated at 37^{0}C for 24 hours. The diameters of the zones of inhibition were measured and reported in mm. the concentrations were repeated 3 times for each of the

bacterium and average diameters were documented. Disks containing 3 µl of dimethyl sulfoxide were used as a negative control.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

MIC values were determined by broth macro dilution assay recommended by the NCCLS (National Committee for Clinical Laboratory Standards, 2002).To determines the minimum inhibitory concentration (MIC) for the *Satureja khuzestaniea* and Thyme essential oils to 10 µl was dissolved in 90 µl DMSO. The final concentration of about 0.1 (mg/ml). In tubes containing 5 ml Mueller Hinton broth samples were cultured and incubated at 37 ° C for 18-24 hours. After incubation for each sample five tubes containing 1ml Mueller Hinton broth were prepared .To each of the tubes ,respectively 1, 2, 3, 4 and 5µl of essential oils were added. After mixing, add bacterial suspension was to final volume 5 × 10 6 CFU/ ml then were incubated at 37 ° C for 18-24 hours and the results were evaluated. In the series of experiments as a positive

Table-1. The essential oil components of Satureja khuzestaniea

% [c]	RI [b]	RT [a]	Compound
0.28	935	4.081	α-pinene
0.39	990	5.158	β-Myrcene
0.49	1016	5.77	α-Terpinene
3.11	1023	5.964	P-Cymene
0.19	1027	6.067	β-Phellandrene
1.24	1056	6.828	ϒ-Terpinene
0.91	1098	7.944	Linalool
0.35	1162	9.89	Borneol
0.65	1173	10.26	Terpinene-4-01
0.19	1291	13.992	Thymol
90.88	1296	14.164	Carvacrol
0.15	1413	17.757	(z)-Caryophyllene
0.21	1502	20.464	β-Bisabolene
0.18	1574	22.57	Caryophyllene oxide

RT[a]: Retention time(min), RI[b]: Retention indices deteimind on HP-5MS capillary coium, %[c]: Calculated from TIC data

control tube (without oil) was used. Bacterial growth after incubation time of opacification was assessed visually. Any opacification or clear slight was considered resistance. Minimum concentration of bacteria was not any significant growth as MIC was determined. MBC values were the first tube that showing no growth on solid media.

2.6. Detection of bla^{OXA-23} and DNA Gyrase-A by PCR

Genomic bacterial DNA was extracted from the five strains by boiling a suspension of bacteria to 95°C for 5 min in a final volume of 50 µL of distilled sterile water. After centrifugation at 13 000g, the supernatants were used as DNA templates.

PCR was performed in a standard enzyme Taq DNA polymerase. Designed primers with genscript software for amplify target fragment were aphA6Forward (5′- ATATACAGAGACCACATACA<G>-3′) Reverse (5′ - GTGCCTCCTTATAGATAG<C>-3′), and DNA gyrase-A Forward (5′- AAGGCCGTCCAATCGTGAA<T>-3′), Revers (5′- AACCGTACCAGAAGCTGTC<G>-3′).

The PCR reactions were performed in a final volume of 25 µL containing 12.5 µL Master Mix(1x) and 5 µL of DNA extract(20 pg) , 1 µL F Primer(0.1 - 1µM) , 1 µL R Primer(0.1 - 1µM), 5.5 µL Sterile Deionized Water with Cinnagen kit. The cycles for bla^{OXA-23} gene mixtures were incubated 120 s primary denaturation at 95°C, secondary denaturation for 45 s at 95°C, annealing for 60 s at 53°C and extension 30 s at 72°C, followed by a final extension for 90 s at 72°C. For DNA Gyrase-A gene mixtures were amplified in step primary denaturation for 120 s at 95°C, secondary denaturation for 5 s at 95°C, annealing for 60 s at 51.8°C and extention for 30 s at 72°C, followed by a final extension for 90 s at 72°C that 35cycle was performed. The amplified products were analyzed by electrophoresis on 1% agarose gel (Cinnagen) containing 0.1 g of ethidium bromide per ml in TAE buffer. The PCR product was visualized under UV light and photographed.

2.7. RT–PCR Analysis for aphA6 and DNA Gyrase-A mRNA Detection

Total mRNA was extracted from five isolates of *A. baumannii*. For quantitative evaluation of product obtained from RNA extracted was measured by a spectrophotometer at a wavelength 260 nm. Nano drop spectrophotometer device that follows the principles and high precision in the measurement of the concentration of nucleic acids was used to measure the concentration of the extracted RNA. The reactions for RT-PCR were 5 µl of DNase-treated RNA in a final volume of 25 µL containing 50 mM of Tris–HCl pH 8.3, 75 Mm KCl, 3 mM MgCl2 , 10 mM DTT, 400 µM of each nucleotide,1 µM of the reverse primer, 1 µM of the forward primer and 100 U of M-MLV reverse transcriptase. The reaction mixtures were incubated for 1 h at 42°C, followed by a 5 min incubation at 95°C. 10 µL of the c DNA was used for amplification of specific bla^{OXA-23} mRNA and DNA Gyrase -A. for bla^{OXA-23} m RNA ,120 s initial denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 53°C and 30 s extension at 72°C, and a final extension step for 5 min at 72°C. And a DNA Gyrase-A mRNA 120 s initial denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 51.8°C and 30 s extension at 72°C, and a final extension step of 5 min at 72°C both PCR products were detected on a 1% agarose gel. Each RT–PCR was performed in triplicate.

Table-2. Results MIC (µg/ml) Satureja khuzestaniea and Thyme essential oil and Zone of growth inhibition(mm) Acinetobacter baumannii

Pathogenic strains	*Satureja khuzestaniea Essence*			*Thyme Essence*			Antimicrobial agents MIC(µg/ml)			Zone of growth inhibition(mm)		
A. baumannii	MIC (µg/ml)	MBC (µg/ml)	Zone of growth inhibition(mm)	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	0.6	0.9	22	>4	>16	>64	0	0	0
A/64	0.3	0.5	24	0.4	0.7	18	>4	>16	>64	0	0	0
A/69	0.5	0.7	30	0.4	0.7	18	>4	>16	>64	0	0	0
A/75	0.2	0.4	25	0.5	0.8	15	>4	>16	>64	0	0	0
A/77	0.2	0.4	15	0.3	0.5	20	>4	>16	>64	0	0	0

CIP(Ciprofloxacin),GM(Gentamaicin),IMP(Imipenem),AMK(Amikacin),CRO(Ceftriaxone),OX(Oxacill n), K(Kanamaycin),N(Neomycin).

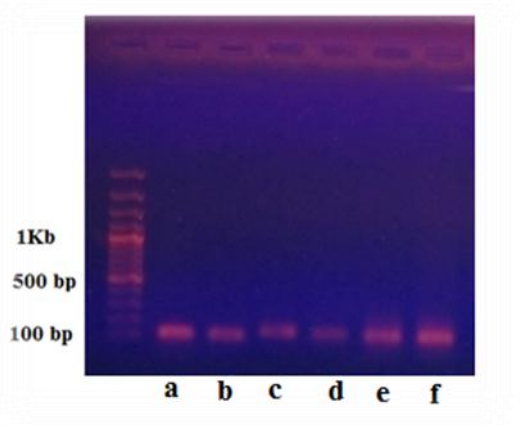

Figure-1. Agar gel electrophoresis results RT-PCR aphA6 and DNA gyrase-A in *Acinetobacter bumannii* strain before and after expose with essential oils.

(a) *Acinetobacter bumannii* strain aphA6 gene before expose with *Satureja khuzestaniea* essence,(b) *Acinetobacter bumannii* strain aphA6 gene before expose with *Thyme* essence ,(c) *Acinetobacter bumannii* strain after the face with Thyme essence,(d) *Acinetobacter bumannii* strain after the face with *Satureja khuzestaniea* essence (e) DNA gyrase A gene before the face with essential oils,(f) DNA gyrase A gene after the face with essential oils.

3. RESULTS

3.1. Chemical Composition of Essential Oil

Forty two compounds were identified, representing 99.8% of the total oil. The major components were carvacrol (90.88%), ρ-cymene (3.11%), and γ terpinene (1.24%), linalool (0.91%). (Table1) and the major compounds in the essential oil of Thyme are thymol (28.8%) and carvacrol (23.46%).

3.2. Antimicrobial Susceptibility Testing

Antibiotic susceptibility test results the number of seventy five Acinetobacter baumanniistrains are as follows. Oxacillin (100%), Amikacin (75%) , Kanamycin (68%), Gentamicin(60%), Imipenem (60%) and (89%) were resistant Neomycin. Inhibitory effects of Satureja khuzestaniea on drug-resistant strains were found. Results MIC (µg/ml) Satureja khuzestaniea and Thyme essential oils and Zone of growth inhibition (mm) Pathogen strain Acinetobacter baumannii (Table 2).

4. CONCLUSIONS

Nosocomial infections caused by Multidrug resistant strains of Acinetobacter baumannii (MDR-AB) are currently among the most difficult to treatment, and they continue to present serious challenges to clinicians' empirical and therapeutic decisions in burned patient (Yali *et al*., 2013).Outbreaks of extensively, and pan drug-resistant A. baumannii (XDR, and PDR, respectively) currently has been reported from worldwide. In this study, the high prevalence of XDR and PDR A. baumannii isolates (37.1% and 8.1%, respectively) from burned patients, is consistent with previous reports (Chang *et al*., 2012). Increasing prevalence of XDR and PDR A. baumannii strains and limited treatment options has prompted the use of antibiotics combinations like tigecycline and colistin as therapeutic regimens [13, 14]. The marker is based on the bacterial aphA6 gene that encodes an aminoglycoside phosphotransferase (APH(3')) and mediates resistance to Kanamycin and Amikacin in Acinetobacter baumannii (Lambert *et al*., 1990).

Their antimicrobial activity is mainly attributed to the presence of some active constituents in their EOs together with their hydrophobicity which enables themfor rupturing cell membranes and intrastructures. Inthis study, satureja essence were used to assess their antibacterial activity against Baumanii. . In this study using satureja essence against these pathogens resulted in these which can be effective enough to reduce the rate of infection.

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