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In-Vitro Antimicrobial Activity and Chemical Composition of *Satureja khuzestanica* Jamzad Essential Oils Against Multidrug-Resistant *Acinetobacter baumannii*

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Abstract

Background: The outbreaks of multidrug-resistant *Acinetobacter baumannii* related to nosocomial infections the organism which the leading cause of mortality in hospitalized patients. Therefore, exploration for alternative antibacterial agents, essential oils have become of major interest.

Objectives: This study aimed to determine the effect of *Satureja khuzestanica* Jamzad essential oil on multidrug-resistant nosocomial isolates of *A. baumannii*.

Methods: Twenty one non-repetitive multidtug-resistant isolates of *A. baumannii* were collected in 2014 from Imam Hossein and Shahid Motahari Burn hospitals in Tehran. Antibacterial susceptibility to 12 antibiotics was measured by disc diffusion. Essential oil extraction of *S. khuzestanica* aerial parts was carried out with Hydro-distillation, and susceptibility to the oil was initially determined using discs containing 1.64 mg essential oil in 10% dimethyl sulfoxide. Minimum inhibitory and bactericidal concentrations of the essential oil were determined by broth microdilution.

Results: The disc diffusion results showed that all isolates were resistant to nine of the 12 antibiotics test which is determined as multidrug-resistant. The disc diffusion results for *S. khuzistanica* essential oil were revealed inhibition zones of 29 - 42 mm. MIC values were 0.31 mg/mL for all test isolates and MBCs were from 0.31 to 0.62 mg/mL which shows the bactericidal activity of the essential oil.

Conclusions: The carvacrol-rich essential oil of *S. khuzistanica* showed strong antibacterial activity against all multidtug-resistant as clinical isolates of *A. baumannii*.

Keywords: Multidrug-Resistance, Essential Oil, *Satureja Khuzestanica* Jamzad

1. Background

Acinetobacter baumannii has become an important cause of nosocomial infections in hospital which outbreaks during the past few decades (1). The target of this organism usually is ill patients in intensive care units (ICU) who resistant to most commonly used antibiotics (2). The remarkable ability of *A. baumannii* to accumulate diverse antibiotic resistance mechanisms has led to the emergence of strains with a broad range of resistance to all existing antibiotic classes and causes a serious concern in clinical practice (2-4). Among the resistance mechanisms, a number of metallo-beta-lactamases and extended-spectrum beta-lactamases production is well documented in clinical isolates of *A. baumannii* (5-8) Therefore, investigating for effective and alternative antibacterial agents such as plant products has been increased. Essential oils which contain high levels of monoterpens are particularly interest, since their lipophilic nature allows them in order to pass through the bacterial cell walls and cytoplasmic membranes. As a result, they can cause cellular content leakage, cell death by disruption of the cytoplasmic membrane, or reaching to other intracellular targets for their antimicrobial activity (9, 10).

Satureja khuzestanica Jamzad is an endemic plant from Southwestern Iran. The essential oil of this plant has been reported for a number of biological activities, including: antibacterial, antifungal, anti-leishmanial, anticancer, antioxidant, anti-inflammatory, anti-diabetic, antihyperlipidemic and antispasmodic properties (11, 12). The antibacterial activity of *S. khuzestanica* essential oil has been shown against a number of bacteria including Gram-positive food pathogens, some members of Enterobacteriaceae, as well as *Pseudomonas aeruginosa* (13, 14). Other recent studies have been shown that carvacrol-rich *S. khuzestanica* EO has antimicrobial activity against clinical urinary isolates of *E. coli*, a number of Gram-positive cocci and yeast strains (15, 16). Also the effect of *S. khuzestanica* EO has been

shown on the expression of the *bap* gene which is involved in biofilm formation and bla_{OXA-23}gene in antibiotic resistant *A. baumannii* using polymerase chain reaction (17, 18).

2. Objectives

This study aimed to determine the effect of *Satureja khuzestanica* Jamzad essential oil on multidrug-resistant (MDR) nosocomial isolates of *A. baumannii*.

3. Methods

3.1. Preparation of S. khuzestanica Essential Oil

The aerial parts of *S. khuzestanica* were collected from Khorramabad (Lorestan province, South of Iran), which is verified by Dr. Sonboli and was deposited with a voucher specimen code of MPH-352 at medicinal plants and drug research institute, Shahid Beheshti University. A Clevenger type apparatus was used for hydro-distillation of the powdered plant aerial parts (250 g) for 3 hours which is recommended by the European Pharmacopoeia. Drying of the obtained essential oil was carried out over anhydrous sodium sulfate before storing at 4°C. The EO was resuspended with dimethyl sulfoxide (1% DMSO) before using in test assays.

3.2. Essential Oil Components Identification

The analysis of the EO was carried out by gas chromatography-flame ionization detection (GC-FID) using a Finnigan system (Thermoquest, Manchester, UK) by a 60 m $\times$ 0.25 mm with 0.25 μm film thickness DB-5 fused silica column (J&W Scientific, Folsom, CA). The carrier gas (nitrogen) was used at a constant flow of 1.1 mL/min with a split ratio of 1:50. The raise of oven temperature is occurred at a rate of 5°C/min from 60°C to 250°C and the temperatures of the injector and detector (FID) were kept at 250°C and 280°C, respectively. Mass spectroscopy (GC-MS) was performed using a Thermoquest Trace GC-MS instrument with the column and temperature program which is described above with the temperature of transfer line at 250°C. The carrier gas (Helium) was used at a flow rate of 1.1 mL/min with a 1:50 split ratio. The components of the EO were identified with their retention indices using same temperature-programmed conditions for n-alkanes and DB-5 column. The EO individual compounds were identified by comparing their mass spectra with reference mass spectra library or authentic compounds, as well as comparing their retention indices with authentic or literature cited compounds. FID area percentages were used in order to obtain semi-quantitative data without using correction factors (12).

3.3. Bacterial Isolates

Twenty one non-repetitive MDR isolates of *A. baumannii* (11 ICU and 10 burn isolates) was collected in 2014 from Imam Hossein and Shahid Motahari Burn hospitals in Tehran. The ATCC standards (*E. coli* 25922, *K. pneumoniae* 10031 and *P. aeruginosa* 85327) were used as susceptibility controls.

3.4. Antimicrobial Susceptibility

According to the CLSI guidelines the disc diffusion method was used in order to confirm the susceptibility of isolates to aztreonam (30 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), piperacillin (100 μg) and piperacillin-tazobactam (110 μg) (MAST, UK) (19). The isolates which were resistant to $>$ 3 antibiotic classes were defined as multidrug-resistant (MDR). Also, the disc method was used in order to determine the susceptibility of the bacterial isolates to the oil using discs containing of 1.64 mg essential oil in 1% DMSO. Negative control discs that only contained DMSO were also included. Minimum inhibitory concentrations (MICs) of the essential oil were determined with broth microdilution as recommended by CLSI (20). Fifty μL of serial two-fold dilutions of each essential oil concentration (0.019 - 5 mg/mL) in Muller Hinton broth (MHB) were added to the wells of 96 well flat bottomed microtiter plates. Fresh bacterial overnight grown were cultured in MHB (50 μL) at 5 $\times$ 10^5/mL. Then, it is added to four wells for each test bacterium, before incubating the microplates at 37°C for 24 hours. The lowest EO concentration which inhibited growth was recorded as MIC. MBC was measured with inoculating 10 μL of the wells contents without bacterial growth on nutrient agar plates before incubation at 37°C for 24 hours. MBC was reported if the colony counts were $<$ 5.

4. Results

Qualitative and quantitative analysis of *S. khuzestanica* EO are presented in Table 1. Nineteen compounds were presented among which carvacrol was the main component (92.87%) followed by limonene (1.2%) (12).

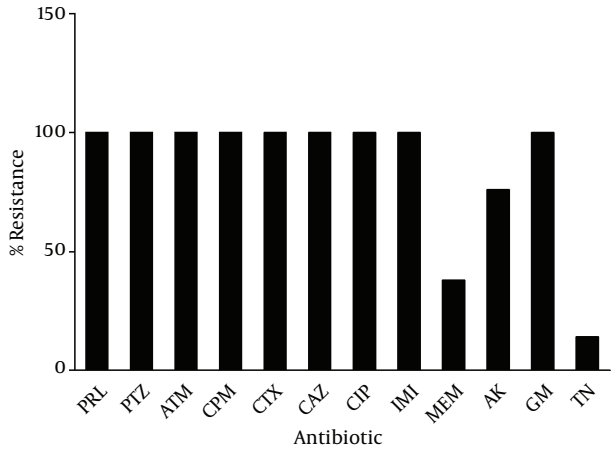
Antibiotic disc susceptibility results showed that all test isolates were multidrug resistant (Figure 1). All of isolates were resistant to ceftazidime, piperacillin, piperacillin-tazobactam, cefepime, cefotaxime, azetronam, imipenem, gentamicin and ciprofloxacin. The susceptibility to other antibiotics was: amikacin, 76%; meropenem, 38%; and tobramycin,

Table 1. Chemical Composition of the Essential Oil of *S. khuzistanica*

Component	RI	%	ID Method
α-Thujene	925	0.21	RI, MS
α-Pinene	933	0.18	RI, MS, CO
Myrcene	981	0.16	RI, MS
α-Terpinene	1013	0.40	RI, MS, CO
p-Cymene	1017	0.51	RI, MS, CO
Limonene	1026	1.20	RI, MS, CO
Z-β-Ocimene	1036	0.18	RI, MS
γ-Terpinene	1053	0.52	RI, MS, CO
trans-Sabinene hydrate	1081	0.57	RI, MS
Terpin-4-ol	1163	0.27	RI, MS
α-Terpinole	1175	0.14	RI, MS
Thymol	1266	0.11	RI, MS, CO
Carvacrol	1282	92.87	RI, MS, CO
Thymyl acetate	1329	t	RI, MS
β-Caryophyllene	1425	0.40	RI, MS, CO
α-Humulene	1427	0.22	RI, MS
β-Bisabolene	1501	0.14	RI, MS
trans-β-Bisabolene	1522	t	RI, MS
TOTAL		98.08	

Abbreviations: CO, co-injection with authentic compounds; MS, mass spectroscopy; RI, retention indices relative to C6-C24 n-alkanes on a DB-5 column; t, trace i.e., < 0.1% by MS, < 0.05% by CO.

Figure 1. Antibiotic Susceptibility of Twenty One *Acinetobacter baumannii* Clinical Isolates



Aztreonam (ATM), amikacin (AK), gentamicin (GM), tobramycin (TN), cefepime (CPM), cefotaxime (CTX), ceftazidime (CAZ), ciprofloxacin (CIP), imipenem (IMP), meropenem (MEM), piperacillin (PRL) and piperacillin-tazobactam (PTZ).

14%. As shown, tobramycin and meopenem had the highest antibacterial activity against our MDR isolates of *A. baumannii*.

The disc diffusion results for *S. khuzistanica* essential oil is revealed inhibition zones of 29 - 42 mm (Table 2). Both of ICU and burn isolates were similarly susceptible to the essential oil. The ATCC standards were also susceptible to the essential oil with the highest inhibition zone for *P. aeruginosa* (43 mm) followed by *E. coli* (26 mm) and *K. pneumoniae* (20 mm). The MIC values for the essential oil were 0.31 mg/mL for all test isolates and 0.62 mg/mL for ATCC susceptible standards. The MBCs were 0.31 - 0.62 mg/mL for *A. baumannii* test isolates and 0.62 mg/mL for ATCC standards showing the bactericidal activity of the essential oil (Table 1).

5. Discussion

The antibacterial activity of *S. khuzistanica* essential oil has been shown against a number of bacterial pathogens. Akbari-Shahabi observed that *S. khuzistanica* essential oil was active against Listeria monocytogenes, an important food pathogen (14). Abbasi and coworkers showed the antibacterial activity of *S. khuzistanica* essential oil against MDR *Pseudomonas aeruginosa*, an important opportunistic pathogen which is responsible for outbreaks of nosocomial infections (13). Ghodrati et al. has been demonstrated the activity of *S. khuzistanica* essential oil against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Staphylococcus epidermidis* and *Candida albicans* (11). In literature search, recent report showing that the antibacterial activity of *S. khuzistanica* essential oil against *A. baumannii* was related to reduce the *bap* gene that involved in biofilm formation by the organism (17). Another recent study showed that *Satureja khuzestanica* Jamzad EO has inhibitory effects on the expression of bla_{OXA-23}gene in drug-resistant *A. baumannii* (18). We believe that our results present the first report on the susceptibility of drug-resistant *A. baumannii* nosocomial strains to *S. khuzistanica* EO. Our results are important since in the recent decades, which *A. baumannii* infections have become a major cause of mortality in hospitalized patients, especially in ICUs and burn wards (21, 22). Azimi et al. has been reported 12% mortality rate in Motahari Burn hospital in 2011, where at least 1 positive *A. baumannii* culture was recovered from all patients (23).

In this study, the essential oil of *S. khuzestanica* contained almost 93% carvacrol as the major component. Also other investigators have reported over 90% carvacrol content in the *S. khuzestanica* essential oils which is collected from different areas of Iran (11, 15, 24, 25). The antimicrobial activity of carvacrol, a phenolic monoterpene has

Table 2. Susceptibility of MDR Clinical Isolates of *A. baumannii* to *S. khuzestanica* EO Measured by Disc Diffusion, MIC and MBC Determinations[a]

A. baumannii Isolates	Disc Inhibition Zone, mm	MIC, mg/mL	MBC, mg/mL
1 NB	37 ± 5.6	0.31	0.31
2 NB	30 ± 0.0	0.31	0.62
5 NB	30 ± 4.2	0.31	0.62
7 NB	31 ± 0.7	0.31	0.31
12 NB	29 ± 1.4	0.31	0.62
13 NB	31 ± 2.1	0.31	0.31
14 NB	30 ± 1.4	0.31	0.62
15 NB	31 ± 2.8	0.31	0.31
35 NB	33 ± 1.4	0.31	0.31
36 NB	37 ± 4.2	0.31	0.31
37 NB	32 ± 4.2	0.31	0.31
26 B	32 ± 2.1	0.31	0.31
27 B	33 ± 1.4	0.31	0.62
28 B	32 ± 1.4	0.31	0.31
29 B	31 ± 1.4	0.31	0.62
31 B	33 ± 2.1	0.31	0.31
33 B	34 ± 0.7	0.31	0.62
47 B	42 ± 0.7	0.31	0.31
54 B	37 ± 4.2	0.31	0.62
55 B	40 ± 0.0	0.31	0.62
58 B	36 ± 2.1	0.31	0.31
***E. coli*[b]**	26 ± 1.4	0.62	0.62
***K. pneumoniae*[b]**	20 ± 0.0	0.62	0.62
***P. aeruginosa*[b]**	43 ± 1.4	0.62	0.62

Abbreviations: B, burn; NB, non-burn ICU isolates.
[a]The numbers assigned to each isolate represents the sequence in which they were obtained from patients.
[b]ATCC standards.

been shown that be related to the presence of a free hydroxyl group and their hydrophobic nature essential for damaging cell membranes (26). Xu et al showed the carvacrol and thymol had the ability in order to permeabilize and depolarize the cytoplasmic membrane of *E. coli* (27). Cristani et al reported that among four tested monoterpnes, carvacrol caused a gross perturbation of the lipid fraction in bacterial cytoplasmic membranes, depending on the lipid composition and the net surface charge of membrane (28). Ultee and coworkers showed that carvacrol changes the permeability of *B. cereus* membrane by dissipation of H^+ and K^+ ion gradients that leads to cell death (29). Di Pasqua et al. reported that carvacrol along with some other monoterpenes, leads to decrease the unsaturated fatty acids in the membrane of treated cells and exert their antimicrobial activities by alterations of the cell envelope (30).

The strong antibacterial activity of *S. khuzistanica* Jamzad essential oil is most probably related to its carvacrol content. Meanwhile limonene, the other major component of our *S. khuzistanica* EO (1.2%), has been reported to have weak or no antibacterial activity against bacterial pathogens (31). In conclusion, carvacrol rich essential oil of *S. khuzistanica* Jamzad showed the strong antibacterial activity against MDR clinical isolates of *A. baumannii* which can be used for treatment of infections in order to eradicate these pathogens. Further research employing a larger number of bacterial isolates is needed to confirm the effectiveness of *S. khuzistanica* Jamzad EO as an anti-infective agent.

Footnotes

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Conflict of Interest: None declared.

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