Original article

Antibacterial activity of *Zataria multiflora* Boiss essential oil against extended spectrum β lactamase produced by urinary isolates of *Klebsiella pneumonia*

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How to cite this article:
Eftekhar E, Zamani S, Yusefzadi M, Hadian J, Nejad Ebrahimi S. Antibacterial activity of *Zataria multiflora* Boiss essential oil against extended spectrum β lactamase produced by urinary isolates of *Klebsiella pneumonia.* Jundishapur J Microbiol. 2011; 4(Supplement 1): S43-S9.

Received: August 2010 **Accepted:** November 2010

Abstract

Introduction and objective: *Klebsiella pneumoniae* is an opportunistic pathogen most frequently associated with extended spectrum β-lactamase (ESBL) production. These organisms are usually resistant to most antibiotics and pose a serious threat for health care associated infections. Plant essential oils rich in carvacrol and thymol have gained importance for their antimicrobial activity. We determined the composition of *Zataria multiflora* essential oil of the Jandagh area in Iran and measured its activity against ESBL producing urinary isolates of *K. pneumoniae*.

Materials and methods: Essential oil was prepared from *Z. multiflora* at full flowering stage by hydrodistillation and its constituents were analyzed by a combination of capillary GC and GC-MS. Antibacterial activity was measured against 10 ESBL producing urinary isolates of *K. pneumoniae* as well as six ATCC bacterial standards by disc diffusion, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) using broth microdilution.

Results: *Zataria multiflora* essential oil contained 25 constituents of which the major components were carvacrol (50.57%), thymol (13.38%) and p-cymene (8.27%). All tested bacteria were susceptible to the essential oil with the exception of *Pseudomonas aeruginosa*. Disc diffusion results showed inhibition zones of 18.3-30.3mm for the ATCC standards and 20.7- 29.7mm for the 10 clinical isolates. MIC and MBC values were 0.015- 2.0mg/ml for ATCC strains and 0.03 to 0.5mg/ml for the clinical isolates.

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Conclusion: *Zataria multiflora* may have the potential to be used against multidrug resistant organisms such as clinical isolates of ESBL producing *K. pneumoniae*.
Keywords: Essential oil; *Zataria multiflora*; Antibacterial activity; Extended spectrum β-lactamase (ESBL); *Klebsiella pneumonia*

Introduction

Extended spectrum beta lactamases (ESBL) mediate resistance to broad-spectrum cephalosporins and are important causes of multidrug resistance in Gram-negative bacteria [1,2]. Among these, *Escherichia coli* and *Klebsiella pneumoniae* are most frequently associated with ESBL production [3]. *K. pneumoniae* is an opportunistic pathogen that causes a significant proportion of community and hospital acquired infections including urinary tract, pneumonia, septicemia and soft tissue infections [4].

Studies have shown that ESBL producing *K. pneumoniae* have rapidly spread worldwide and pose a serious threat for health care associated infections [5]. The occurrence of ESBL producers among *K. pneumoniae* isolates collected over three years was shown to be highest in India (72%), followed by Mexico (71.4%), Latin America (37.8%-55.3%), Greece (43.1%), Poland (37.5%), Asia/Pacific Rim (22.4%), Europe (13.3%) and North America (7.5%) [6]. Prevalence of ESBL producing *K. pneumoniae* in Iran has been reported to range from19.6% to 75.75% [7-9]. These alarming data show the necessity of seeking alternative antibacterial agents such as plant natural products.

Plant products have been recognized as antimicrobial agents for some time [10]. Among these, few plants have been reported for their biological activities against multidrug resistant bacteria including the ESBL producing *E. coli* and *K. pneumoniae* [11-13]. *Zataria multiflora* Boiss (Avishan-e-Shirazi in Persian and Sa'atar or Zaatar in the old Iranian medical books) is a thyme-like plant and a member of *Labiatae* family that grows wild in central and southern parts of Iran [14]. It is used in traditional folk remedies for its antiseptic, analgesic (pain-relieving) and carminative (anti-flatulence and intestine-soothing) properties [14,15].

The antibacterial activity of *Z. multiflora* has been shown against a number of Gram-positive and Gram-negative bacteria [16-20]. However, there is no report on its activity against multidrug resistant or ESBL producing organisms. We determined the composition and antibacterial activity of the essential oil of *Z. multiflora* against ESBL producing urinary isolates of *K. pneumoniae* as well as ATCC bacterial standards.

Materials and methods

Plant material and essential oil preparation

The aerial parts of *Z. multiflora* were collected at full flowering stage from Jandagh in Isfehan province at an altitude of 1230m. A voucher specimen was deposited at the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. The essential oil was prepared by hydrodistillation and the major oil components were analyzed by a combination of capillary gas chromatog-raphy (GC) and gas chromatography-mass spectroscopy GC-MS.

Essential oil analysis

Gas chromatography-flame ionization detector (GC-FID) analyses of the oil were conducted using a Thermoquest-Finnigan instrument (Thermo Fisher Scientific, USA) equipped with a DB-5 fused silica column (60m × 0.25mm i.d., film thickness 0.25µm). Nitrogen was used as the carrier

gas at the constant flow of 1.1ml/min. The split ratio was 1/50. The oven temperature was raised from 60ºC to 250ºC at a rate of 5ºC/min. The injector and detector (FID) temperatures were kept at 250ºC and 280ºC, respectively. GC-MS analysis was carried out on a Thermoquest-Finnigan Trace GC-MS instrument equipped (Thermo Fisher Scientific, USA) with the same column and temperature programming as mentioned for GC. Transfer line temperature was 250ºC. Helium was used as the carrier gas at a flow rate of 1.1ml/min with a split ratio equal to 1/50.

Oil components identification

The constituents of the essential oils were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C_6-C_{24}) and the oil on a DB-5 column under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Wiley 7.0) or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature [21]. Semi-quantitative data was obtained from FID area percentages without the use of correction factors.

Bacterial strains

Ten multidrug resistant, imipenem sensitive ESBL produced by urinary isolates of *K. pneumoniae* were chosen from a bacterial collection maintained in the microbiology research laboratory at Shahid Beheshti University. In addition, *Bacillus subtilis* (ATCC 465), *E. coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29737), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 85327) and *Staphylococcus aureus* (ATCC 25923) were also employed.

Antibacterial susceptibility measured by disc diffusion

The antibacterial activity of the essential oil was determined by disc diffusion according to NCCLS guidelines [22]. Briefly, 0.1ml of a suspension of the test microorganism (10^8cells/ml) was spread on Mueller-Hinton agar (Merck, Germany) plates and sterile 6mm discs, each containing 7.2mg of essential oil were placed on the microbial lawns. Antibiotic discs including gentamycin (10µg), tetracycline (30µg) and imipenem (10µg), (Padtan Teb, Tehran, Iran) were also included. The tests were carried out in triplicate and plates were incubated at 37ºC for 24h. The diameters of inhibition zones were measured following the incubation period and reported in mm.

Determination of minimum inhibitory concentrations and minimum bactericidal concentrations

Minimum inhibitory concentrations (MIC) values were determined by broth microdilution as recommended by NCCLS [23]. Serial two-fold dilutions of the essential oil within the range of 0.062-64mg/ml were made in Mueller-Hinton broth (MHB) [Merck, Germany] containing 0.5% Tween 80 in 96-well microtiter plates. Fresh bacterial suspensions, prepared from overnight grown cultures in MHB were added to give a final concentration of 5×10^5 organisms/ml.

Controls of bacteria or the essential oil alone were also included. The microplates were incubated at 37ºC for 24h and the first dilution with no growth was recorded as MIC. Minimal bactericidal concentrations (MBC) were determined by spreading 100µl of the contents of the MIC wells that showed no bacterial growth on nutrient agar plates followed by incubation at 37ºC for 24h. The first well with colony counts of <5 was considered to be negative for growth and reported as the MBC.

Results

Based on the dry weight, the hydrodistillition of the aerial parts of *Z. multiflora* yielded 2.5% (w/w) oil representing 97.98% of the total oil. The composition of the oil from *Z. multiflora* is shown in table 1. As shown, carvacrol was the major component constituting 50.57% followed by thymol (13.10%) and p-cymene (8.27 %). The results of the antibacterial activity of *Z. multiflora* essential oil by the disc diffusion assay are given in table 2. As observed, all clinical isolates as well as the ATCC standards (except for *P. aeruginosa*) showed remarkable susceptibility compared to the zones obtained with the antibiotic discs. The inhibition zones ranged from 18.3- 30.3mm for the ATCC standards and 20.7- 29.7mm for the 10 clinical isolates. These results are significant considering the fact that all clinical isolates were multidrug resistant ESBL producers.

As it is shown in table 2, the oil MIC values for the ATCC standards were within the range of 0.015- 2.0mg/ml except for *P. aeruginosa* where a value of 32mg/ml was obtained. Among the ATCC standards, *B. subtilis* and *S. aureus* (MIC of 0.015) were the most sensitive organisms followed by *E. coli* (MIC of 0.25mg/ml), *K. pneumoniae* (MIC of 0.5mg/ml) and *E. faecalis* (MIC of 2mg/ml). As it is also shown in table 2, MIC values were 0.5mg/ml for one clinical isolate, 0.25mg/ml for three, 0.125mg/ml for two, 0.062mg/ml for two and 0.031mg/ml for two isolates. Comparison of MIC and MBC results indicate the bactericidal nature of the essential oil.

Table 1: Composition of *Z. multiflora* essential oil

Compounds	RI	%
α-thujene	927.27	0.08
α-pinene	936.74	2.00
camphene	950.76	0.09
3-octanone	965.91	0.18
β-pinene	978.03	0.16
mycerene	982.95	0.68
ρ-cymene	1017.5	8.27
β-terpineol	1026.0	0.9
γ-terpinen	1053.1	2.84
linalool	1085.7	1.27
p-menth-1-en-4-ol	1168.2	1.04
p-menth-1-en-8-ol	1180.3	1.12
carvacrol methyl ether	1228.0	1.62
thymol	1268.7	13.10
carvacrol	1284.7	50.57
thymyl acetate	1329.7	0.68
carvacryl acetate	1348.9	3.83
trans-caryophyllene	1431.0	3.5
eudema-3,7-dien	1448.0	0.1
aromadendrene	1451.2	2.03
α-humulene	1463.5	0.2
cyclosativene	1471.0	0.12
ledene	1502.9	1.07
spathulenol	1577.3	1.08
caryophylene oxide	1584.7	1.45
Monoterpene hydrocarbons		11.46
Oxygenated monoterpenes		76.97
sesquiterpene hydrocarbons		7.02
oxygenated sesquiterpenes		2.53
other		
total		97.98

Compounds listed in the order of their elution from a DB-1 column. RI, retention index relative to *n*-alkanes (C_6- C_{24})

Table 2: Antibacterial activity of *Z. multiflora* essential oil by disc diffusion, MIC and MBC determinations

Microorganism	Inhibition Zone (mm)				μg/ml oil	
	Oil	GM	TC	IMP	MIC[a]	MBC[b]
B. subtilis *	30.0 ±1.0	23	30	nt	0.015	0.015
E. faecalis *	18.3±0.6	6.0	11	nt	2.01	2.01
S. aureus *	30.0±0.1	17	17	nt	0.015	0.03
E. coli *	24.7±0.1	20	20	nt	0.25	0.25
*K. pneumoniae**	24.3±1.5	20	24	nt	0.50	0.50
P. aeruginosa *	6.00 ±0.0	20	20	nt	32.0	32.0
K. pneumoniae UI 9	20.7±0.6	13.3±0.6	nt	17.3±1.2	0.25	0.25
K. pneumoniae UI 10	21.3±0.6	12.7±0.6	nt	17.3±0.6	0.125	0.125
K. pneumoniae UI 13	29.7±1.1	16.3±1.5	nt	23.7±0.6	0.031	0.031
K. pneumoniae UI 14	23.0±1.0	13.7±0.6	nt	18.3±0.6	0.125	0.125
K. pneumoniae UI 17	22.7±1.1	15.3±1.5	nt	20.3±0.6	0.25	0.25
K. pneumoniae UI 33	24.7±0.6	13.5±0.7	nt	19.3±0.6	0.031	0.031
K. pneumoniae UI 41	20.7±0.6	15.0±0.0	nt	18.0±1.0	0.50	0.50
K. pneumoniae UI 43	22.3±0.6	15.3±0.6	nt	22.0±1.4	0.25	0.25
K. pneumoniae UI 46	23.7+1.5	15.0+0.0	nt	18.5±0.7	0.062	0.062
K. pneumoniae UI 54	23.7+0.6	9.0+0.0	nt	16.0±0.0	0.062	0.062

[a]. Minimum inhibitory concentration: [b.] Minimum bactericidal concentration: UI, urinary isolate: nt, not tested: *, ATCC standards. GM, gentamycin: TC, tetracycline: IMP, imipenem. Values are means of 3 repeats ± standard deviations

Discussion

The composition of the essential oil and various organic extracts of *Z. multiflora* have been studied and the major oil constituents were reported mostly as carvacrol and in some studies, thymol [16-18,24]. In our hands, analysis of *Z. multiflora* essential oil showed carvarol to be the major oil component constituting over 50% of the oil, higher than those reported in other studies.

Essential oils rich in carvacrol and thymol have recently gained increasing importance for their considerable antimicrobial and antioxidant activity [25, 26]. Among the phenolic compounds, carvacrol has been shown to have the highest antimicrobial activity due to its hydrophobic nature and the presence of a free hydroxyl group which is essential for its activity on cell membranes [27,28]. The antibacterial activity of *Z. multiflora* has been reported against a range of microorganisms including the Gram-negative enteric bacteria [18-21]. However, most studies have shown the biological activity of the plant extracts and essential oil against susceptible bacterial standard cultures.

We showed that *Z. multiflora* essential oil had a considerable *in vitro* activity against the standard ATCC cultures (except for *P. aeruginosa*) as well as the ESBL producing clinical isolates of *K. pneumoniae*. In fact, the MICs obtained against the clinical isolates were equal or lower than those obtained for the standard ATCC cultures. Reports on plant antibacterial activities against ESBL producing bacteria are rare and few studies

have been carried out using ESBL producing *E. coli* and *K. pneumoniae* [11-13].

We believe that this is the first report on the biological activity of *Z. multiflora* against ESBL producing clinical isolates of *K. pneumonia*. The increasing rate of ESBL production in *K. pneumoniae* clinical isolates is alarming and the fact that most of these organisms are multidrug resistant causes serious concern in eradication of these infections. Considering that few antibiotics could be used for the treatment of ESBL producing *K. pneumoniae*, seeking alternative therapeutic agents such as natural plant products are extremely important. Carvacrol and thymol are the most likely candidates for this biological activity as we have previously shown their antibacterial activity against the clinical isolates of *H. pylori* [29].

Conclusion

The strong biological activity of *Z. multiflora* essential oil against ESBL producing multidrug resistant isolates of *K. pneumoniae* suggests that the oil or its effective components may provide an alternative treatment for infections caused by multidrug resistant, ESBL producing *K. pneumoniae*.

Acknowledgments

The authors wish to thank Shahid Beheshti University Research Council for providing a special grant to finance this research.

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