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Chemical composition and antibacterial activity of *Teucrium polium* essential oil against urinary isolates of *Klebsiella pneumoniae*

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The alarming resistance of many bacterial pathogens to most available antibiotics has increased interest in natural plant products as alternative antibacterial agents. In this research, the antibacterial activity of *Teucrium polium* essential oil was determined against urinary isolates of *Klebsiella pneumoniae*. The aerial parts of *T. polium* were collected at full flowering stage in Tehran, Iran. The essential oil was isolated by hydrodistillation and analyzed by a combination of capillary gas chromatography (GC) and gas chromatography–mass spectometry (GC–MS) Antibacterial activity was measured against fifteen clinical isolates of *K. pneumoniae* by disc diffusion. Minimum inhibitory and bactericidal concentrations were also determined using broth microdilution. Twenty constituents were detected in *T. polium* essential oil of which the major components were β-caryophyllene (29%), farnesene (13%), β-pinene (11%) followed by germacrene D (6.5%) and α-pinene (5.5%). All *K. pneumoniae* clinical test isolates were susceptible to the essential oil by disc diffusion with inhibition zones within the range of 14–28.5 mm. Minimum inhibitory and bactericidal concentration values were 0.62–1.25 mg/ml and confirmed the disc test results. *T. polium* essential oil may have a potential for use against multidrug resistant organisms such as clinical isolates of *K. pneumoniae*.

Keywords: essential oil; *Teucrium polium*; Antibacterial activity; *Klebsiella pneumoniae*

Introduction

Klebsiella pneumoniae is an opportunistic pathogen and a common cause of community and hospital acquired infections including urinary tract, pneumonia, septicemia and soft tissue infections (1). In recent years, the alarming bacterial resistance to many available antibiotics has limited the use of these drugs. E.g. fluoroquinolones such as ciprofloxacin (CIP) which previously had excellent activity against clinical isolates of *Klebsiella*, have recently become less effective due to their extensive use causing an increase in the frequency of CIP resistant *K. pneumoniae* worldwide (2, 3). Therefore, interest in alternative sources of antibacterial agents such as plant products has increased. Plants are known to produce a variety of compounds for protection against a number of pathogens and can be considered as natural sources for antibacterial agents (4, 5). We have previously reported the biological activity of *Zataria multiflora*, rich in carvacrol and thymol, against ESBL producing clinical isolates of *K. pneumoniae* (6).

Teucrium polium is a perennial shrub, 20–50 cm high, distributed widely in the dry and stony hills and deserts of almost all Mediterranean countries, Southwestern Asia, Europe, and North Africa (7). The bruised foliage releases a pleasant aromatic odor and the flowers are small, in clusters and range from pink to white. *T. polium*, locally called kalpooreh, is abundant in Iran (8). The genus *Teucrium* is one of the richest sources of neoclerodane diterpenes. Over 220 diterpenes have been described, many of which of interest for their insect-repellent and medicinal properties. Essential oils of *Teucrium* have been the subject of many studies. Depending on the species and the plant origin, the yields of the essential oil (0.05–1.5%) and the amounts of the main constituents (mono and sesquiterpene hydrocarbons, and oxygenated sesquiterpenes) differ widely (9).

In the Mediterranean countries, the plant has been used for a variety of conditions such as gastrointestinal disorders, inflammations, diabetes, and rheumatism (10, 11). In the traditional Iranian medicine, *T. polium* tea is used to treat ailments such as abdominal pain, indigestion, common colds, and urogenital diseases (10). *T. polium* has been also reported to have antibacterial, antiulcer, antispasmodic, anorexic, and antipyretic activities (12–14). Recently, cytotoxic, anticancer, and antimutagenic effects of ethanol and aqueous extracts of *T. polium* have been shown on various cell lines (15–17). In a recent review, different classes of bioactive compounds with a broad spectrum of pharmacological effects including antioxidant, anticancer, anti-inflammatory, hypoglycemic, hepatoprotective, hypolipidemic as well as antibacterial and antifungal

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activities have been examined (18). The aim of this study is to investigate the essential oil composition of the aerial parts of *T. polium* and determine its antibacterial activity against fluoroquinolone resistant urinary isolates of *K. pneumoniae.*

Experimental

Plant material and essential oil preparation

The aerial parts of *T. polium* were collected at full flowering stage from Tehran, Iran, in May 2012. The plant was identified and deposited at the Herbarium of Ecology and Systematic Department, Research Institute of Applied Sciences, Shahid Beheshti University (Tehran), where it was assigned a voucher specimen number (AS-86116). Air-dried aerial parts of the plant (500 g) were hydrodistilled using a Clevenger type apparatus for 4 hours. The resulting essential oils were dried over anhydrous sodium sulfate and stored at 4°C until analyzed and tested.

Essential oil analysis and identification of oil components

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 (60 m × 0.25 mm i.d. film thickness 0.25 μm). Nitrogen was used as the carrier gas at the constant flow of 1.1 ml/min. The split ratio was 1/50. The oven temperature was raised from 60 to 250°C at a rate of 5°C/min. The injector and detector (FID) temperatures were kept at 250 and 280°C, respectively. Gas chromatography–mass spectometry (GC–MS) analysis was carried out on a Thermoquest-Finnigan Trace GC–MS instrument equipped with the same column and temperature programming as mentioned for GC. Transfer line temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1.1 ml/min with a split ratio equal to 1/50.

The constituents of the essential oil were identified by calculation of their retention indices under temperature-programmed conditions for n-alkanes (C6–C24) 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 those reported in the literature (19). Semi-quantitative data were obtained from FID area percentages without the use of correction factors.

Bacterial strains

Fifteen urinary isolates of *K. pneumoniae* were chosen from a bacterial clinical collection maintained in the microbiology research laboratory at Shahid Beheshti University. Bacteria were isolated from urine samples of patients admitted to Imam Hussein hospital in Tehran from July 2010 to January 2011. In addition, *K. pneumoniae* ATCC 10031 was employed as the susceptible control.

Antibacterial susceptibility measured by disc diffusion

The antibacterial activity of the essential oil was determined by disc diffusion according to the CLSI guidelines (20). Briefly, 4–5 colonies of each test strain from overnight grown cultures on nutrient agar were inoculated into 5 ml of Mueller Hinton broth (Liofilchem, Italy) and were incubated at 37°C for 4–6 hours. The turbidity of the cultures was then adjusted to MacFarland Standard 0.5 which corresponds to around 10^8 cells/ml. A 0.1 ml of each suspension was then spread on a Mueller Hinton agar plate (Liofilchem, Italy) and 6 mm sterile discs, each containing 5 mg of essential oil, were placed on the microbial lawns. Antibiotic discs including nalidixic acid (NA) (30 μg) and CIP (5 μg) (Himedia, India) were also included. The tests were carried out in duplicate and plates were incubated at 37°C for 24 hours. The diameters of inhibition zones were measured following the incubation period and reported in mm.

Determination of minimum inhibitory and bactericidal concentrations

Minimum inhibitory concentrations (MIC) values were determined by broth microdilution as recommended by CLSI (21). Serial twofold dilutions of the essential oil were made in Mueller Hinton broth (MHB, Liofilchem, Italy) containing 0.5% DMSO within the range of 0.062–10 mg/ml in ninety-six-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 37°C for 24 hours and the first dilution with no growth was recorded as MIC. Minimal bactericidal concentrations (MBC) were determined by spreading 100 μl of the contents of the MIC wells that showed no bacterial growth on nutrient agar plates followed by incubation at 37°C for 24 hours. The first well with colony counts of <5 was considered to be negative for growth and reported as the MBC.

Results and discussion

Essential oil composition

The essential oil prepared from *T. polium* had a yield of 0.5% on dry weight basis (w/w). Twenty compounds representing 93.6% of the oil were identified. As shown in Table 1, the major components of *T. polium* essential

Table 1. Composition of the essential oil of *Teucrium polinum*.

Compound	RI	% of the oil
α-Thujene	926	0.4
α-Pinene	935	5.5
Sabinene	972	2.2
β-Pinene	976	11
Myrcene	982	0.5
ρ-Cymene	1015	0.1
Limonene	1023	4.2
Borneol	1155	0.1
Terpinene-4-ol	1172	Trace
Bornyl acetate	1280	0.3
α-Copaene	1375	0.4
β-Caryophyllene	1430	29
Farnesene	1445	13
α-Humulene	1460	2.9
Germacrene D	1485	6.5
Bicyclogermacrene	1499	5
β-Bisabolene	1504	3.7
γ-Cadinene	1520	1.1
Spathulenol	1573	1.5
Caryophyllene oxide	1580	0.7
Total		93.6

Notes: Compounds listed in the order of their elution from a DB-1 column. RI; retention index relative to n-alkanes (C6–C24).

oil were β-caryophyllene (29%), farnesene (13%), and β-pinene (11%) followed by germacrene D (6.5%) and α-pinene (5.5%). The essential oil composition of *T. polium* has been the subject of several investigations. In a report from Egypt that dates back to 1974, twelve major compounds were characterized in *T. polium* including ocimene, α-pinene, menthane, and pulegone (22). Vokou and Bessiere (23) identified thirty-five compounds from leaves and flowering heads, mostly sesquiterpenes (α- and τ-cadinols), (E)-β-caryophyllene, and its oxide forms. In a study performed in Turkey, the stem and leaf oils of *T. polium* contained β-pinene, nerolidol, and α-pinene as major oil components (24). An Iranian study by Eikani et al. (25) showed that the major components of *T. polium* oil were sesquiterpenes, mostly β-caryophyllene (18.0%) and germacrene D (13.2%), closer to our results. In another study performed by Moghtader in 2009 (26) on *T. polium* from Kerman province in Iran, twenty-eight compounds were identified and the main constituents were α-pinene (12.52%), linalool (10.63%), caryophyllene oxide (9.69%), β-pinene (7.09%), and β-caryophyllene (6.98%). Overall, most studies on the oil composition of *T. polium* include α and β-pinenes as well as β-caryophyllene as major components. There are a number of reasons which can explain differences between the essential oil components of the same plant. These include: the distribution of numerous subspecies, geographical locations as well as the environmental physiological conditions (18).

Antibacterial activity

The antibacterial activity of *T. polium* essential oil by disc diffusion is shown in Table 2. As observed, all *K. pneumoniae* clinical isolates as well as the ATCC standard were susceptible with inhibition zones ranging from 14 to 28.5 mm. In fact, only two isolates had inhibition zones around 14 mm and the rest revealed much larger zones indicating the high sensitivity of the majority of the clinical isolates to the oil. These results are significant considering the fact that most of the isolates (10/15) were resistant to both naidixic acid CIP by disc diffusion. As shown in Table 2, the oil MIC values for the clinical isolates were within the range of 0.62–1.25 mg/ml. MIC and MBC values confirmed the disc test results and were 0.62 mg/ml for majority of the clinical isolates (13/15) as well as the ATCC standard. These results indicate the bactericidal nature of the *T. polium* essential oil.

The antibacterial activity of *T. polium* has been mostly studied using the organic extracts of plant aerial parts. Autore et al. (12) reported the antibacterial activity of the ethanol extract of the flowering tops against both gram-positive and gram-negative bacteria. Essawi and Srour (27) screened the antibacterial activity of organic and aqueous extracts of *T. polium* aerial parts against eight different bacteria and showed the activity of both extracts against gram-positive and gram-negative bacteria. However, the organic extract showed greater activity compared to the aqueous extract. In another study, Sarac and Ugur (28) investigated the antimicrobial activity of

Table 2. Antibacterial activity of *T. polium* essential oil by disc diffusion, MIC and MBC determinations.

	Inhibition zone (mm)			mg/ml oil	
Bacteria	OIL	NA	CIP	MIC	MBC
K. pneumoniae ATCC 10031	22.0	23	26	0.62	0.62
K. pneumoniae UI 1	14.5	0	6	1.25	2.5
K. pneumoniae UI 27	18.5	22	23	0.62	0.62
K. pneumoniae UI 33	20.0	18	14	0.62	0.62
K. pneumoniae UI 34	21.0	0	0	0.62	0.62
K. pneumoniae UI 44	28.5	0	0	0.62	0.62
K. pneumoniae UI 45	24.5	0	0	0.62	0.62
K. pneumoniae UI 46	27.5	0	0	0.62	0.62
K. pneumoniae UI 67	17.0	0	0	0.62	1.25
K. pneumoniae UI 72	27.0	23	26	0.62	0.62
K. pneumoniae UI 78	19.0	0	0	0.62	0.62
K. pneumoniae UI 86	15.5	22	20	0.62	0.62
K. pneumoniae UI 92	14.0	14	22	0.62	0.62
K. pneumoniae UI 93	22.0	23	25	0.62	0.62
K. pneumoniae UI 94	19.5	20	24	0.62	0.62
K. pneumoniae UI 96	23.0	12	15	0.62	0.62

Notes: MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; UI – urinary isolate. For NA, an inhibition zone of $\geqslant$19 mm and for CIP, a zone of $\geqslant$21 mm determines sensitivity.

T. polium ethanol extract against various test micro-organisms including bacteria that are resistant to multiple antibiotics and found activity against gram-positive bacteria, especially staphylococci. Ilhami et al. (29) examined the activity of *T. polium* acetone and chloroform extracts against eleven bacterial species and showed that both extracts were effective against all test organisms except for *Escherichia coli*. Stankovic et al. (30) reported the antibacterial activity of twenty-one crude extracts from seven taxa of the genus *Teucrium* especially against *Staphylococcus aureus* ATCC 25923. Recently, Djabou et al. (9) suggested that *Teucrium* essential oils have the potential to be used as food preservatives and to prevent the growth of nosocomial bacteria. We believe that this is the first report on the biological activity of *T. polium* essential oil against clinical isolates of *K. pneumoniae.*

The specific role and/or contribution of the individual compounds of *T. polium* are not clear. The antibacterial properties of caryophyllene oxide and *β*-caryophyllene have been shown (31–34). *β* -pinene is also known for both antifungal and antibacterial activity (35). The strong biological activity of *T. polium* essential oil against quinolone resistant isolates of *K. pneumoniae* of this study suggests the potential of the plant as a new source of antibacterial agents for treatment of infections caused by antibiotic resistant *K. pneumoniae* and perhaps other drug resistant gram-negative pathogens. However, further studies are needed to determine the toxicity of the essential oil before its internal use becomes possible.

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