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Chemical composition and antibacterial activity of *Origanum ramonense* essential oil on the β -lactamase and extended-spectrum β -lactamase urinary tract isolates

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Abstract

The aim of this study was to evaluate the antibacterial activity of *Origanum ramonense* essential oil extracted from the air-dried leaves against β -lactamase and extended-spectrum β -lactamase obtained from the patients with urinary tract infection. The essential oil was extracted by hydrodistillation and analyzed by GC-MS. *In vitro* antibacterial activity was studied using disc diffusion and micro-dilution methods. Twenty compounds were identified representing 97.8% of the total oil. The major components were carvacrol (84.6%), p-cymene (4.3%) and γ -terpinene (3.3%). The oil showed a broad spectrum of antibacterial activity against all tested isolates. *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* had the lowest minimum inhibitory concentration values (0.015 μ g/mL) followed by *Escherichia coli* (0.14 μ g/mL). The lowest susceptible strains to oil were *Pseudomonas aeruginosa*, *Proteus mirabilis*, *E. coli* 25922 and *P. aeruginosa* 10145. The bacteriostatic and bactericidal effects at concentrations as low as 0.015 μ g/mL indicated the potent antibacterial activity of *O. ramonense*.

Introduction

Urinary tract infection is one of the most common and highly recurrent bacterial infections. The most prevailing bacteria which are responsible is the *Escherichia coli*. Many other urinary tract infection-causing genera are also isolated from the patients with a variable degree of infection such as *Klebsiella*, *Enterobacter*, *Proteus*, *Serratia* and *Pseudomonas aeruginosa* (Al-Asoufi et al., 2017).

However, a significant number of infections caused by multidrug-resistant bacteria among urinary tract infection patients, such as the extended β -lactamase-resistant bacteria, have been reported (Bartoletti et al., 2016). For instance, antimicrobial resistance survey on cystitis showed that a high percentage of *E. coli* strains were ampicillin, trimethoprim/sulfamethoxazole and nalidixic acid resistant strains while *K. pneumoniae* strains were mecillinam, fosfomycin, cefuroxime and

nitrofurantoin resistant (Schito et al., 2009). In addition, 12% of patient with UPEC urinary tract infection had fluoroquinolone-resistant isolates (Van der Starre et al., 2010). The increasing prevalence of urinary tract infection isolates that were resistant to routinely used antibiotics, particularly to beta-lactams or extended-spectrum beta-lactamase, has stimulated interest in finding new and effective antimicrobial agents from other sources such as plants.

Essential oils have been applied for centuries in pharmaceuticals and alternative medicine. They are effective as antibacterial, antifungal, antiviral, anti-inflammatory, anti-oxidant, and as anti-cancer (Bakkali et al., 2008; Tarawneh et al., 2010; Benelli et al., 2017; Burt, 2004; Martinelli et al., 2017). Essential oils are secondary metabolites produced by aromatic plant families such as Apiaceae and Lamiaceae. They are the mixture of



terpenes (monoterpenes and sesquiterpenes), terpenoids (isoprenoids), and aliphatic and aromatic compounds including aldehydes and phenols (Sakkas and Papadopoulou, 2017).

Origanum is a genus that includes 54 species in the family Lamiaceae. Traditionally, decoction of leaves and stems as well as their essential oils are widely used as remedies of upper respiratory and digestive complaints such as cough, sore throat and gastric spasm. Generally, the ability of *Origanum* species to inhibit the growth of pathogenic bacteria has been investigated in numerous studies. Reports showed that *Origanum* essential oil possesses potential antibacterial activity with low minimum inhibitory concentration and minimum bactericidal concentration (Bassanetti et al., 2017; Carezzano et al., 2017; Habbadi et al., 2017; Özkan et al., 2017). Therefore, they can be a powerful tool to reduce the impact of bacterial drug resistance issue.

To the best of our knowledge, this is the first report on the antibacterial effect and the second one about the chemical composition of *O. ramonense* essential oil. Thus, the aims of this study are to evaluate the antibacterial activity of essential oil extract from the *O. ramonense* leaves against β -lactamase and extended-spectrum β -lactamase obtained from patients with urinary tract infection symptoms and to survey the chemical constituents of the essential oil extract of Jordan habitat *O. ramonense* dried leaves.

Materials and Methods

Plant materials

Areal parts of *O. ramonense* were collected from Aye region, Al-Karak Province, south of Jordan during March to April 2017. The plant was identified by Dr. Feryal Al-Khresat (Department of Biology, Mu'tah University, Al-Karak, Jordan) according to flora Palaestina, part 3. Voucher specimen had been deposited in the Department of Medical Laboratory Sciences, Faculty of Science, Mu'tah University, Al-Karak, Jordan.

The freshly collected materials were air dried at room temperature in the shade and then the leaves were separated from the stem and subjected to essential oil extraction.

Essential oil extraction

Sample of 150 g of the dried leaves was subjected to hydrodistillation using simple Clevenger apparatus for 4 hours. The oil was extracted from the aqueous phase using n-hexane and dried over anhydrous sodium sulfate (Na_2SO_4). Finally, the extracted oil was stored as aliquots at 4°C.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed using Varian chromatograph CP-3800 GC-MS-MS-200 equipped with split-splitless injector, DB-5 GC column and the mass detector was set to scan ions between 40-400 m/z using full scan mode and electron impact (EI, 70 eV). The parameters and conditions used in this test were similar to that used by (Tawaha et al., 2015). A hydrocarbon mixture of n-alkanes (C8-C20) was analyzed separately by GC-MS using the same column (DB-5) and under the same chromatographic conditions. The compounds were identified by comparison of their retention time to n-alkaline retention times and their similarities to mass spectra database (NIST library) and published reports.

Antibacterial activity

Bacterial strains

Seven clinical isolates were used in this study including five Gram negative strains: Extended-spectrum β -lactamase producing *E. coli*, extended-spectrum β -lactamase producing *Proteus mirabilis*, β -lactamase producing *K. pneumoniae*, β -lactamase producing *Pseudomonas aeruginosa* and *E. aerogenes*, and two Gram positive strains: β -lactamase producing *Staphylococcus aureus* and *S. epidermidis*.

The clinical isolates were obtained from the Al-Salam Specialty Hospital (Al-Karak, Jordan) and Al Bashir Hospital (Amman, Jordan). These strains were isolated from patients with urinary tract infection symptoms and they were characterized by BIOMÉRIEUX VITEK® 2 SYSTEM or by Enterosystem 18 R (Liofilchem). In addition, two reference strains: *E. coli* 25922 and *P. aeruginosa* 10145 provided by Dr. Wael Al-Zereini (Department of Biology, Mu'tah University, Al-Karak, Jordan) were used.

Disc diffusion method

The disc diffusion method was performed as previously described (Qaralleh et al., 2010) with some modifications. Briefly, 100 μL of a bacterial suspension containing 1.5×10^8 CFU/mL (0.5 McFarland's standard) was spread using a sterile swab on Mueller-Hinton agar plates. Then, sterile blank discs containing 3 μL essential oil or ciprofloxacin (5 μg) were placed onto the inoculated plates. The plates were incubated at 37°C for 24 hours. Each test was performed in triplicate and the zone of inhibition was measured as millimeter diameter.

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration was measured using the microdilution method with some modifications (Qaralleh et al., 2010). Three-fold dilution was

prepared using 96 well plats from a stock solution of 10 μg essential oil in 10% DMSO solvent. Then 10 μL of bacterial suspension containing 1.5×10^8 CFU/mL (0.5 McFarland's standard) was inoculated into each well. The same test was carried out with 10% DMSO as a control. Each test was performed in triplicate. The lowest concentration of essential oil needed to inhibit the visible growth of the tested microbes after 24 hours was considered as the minimum inhibitory concentration value.

The minimum bactericidal concentration was determined by sub-culturing and spreading the wells content with concentrations of equal to and higher than the minimum inhibitory concentration value on agar plate. The lowest concentration of essential oil that exhibited no growth on the agar plate after incubation at 37°C for 24 hours was reported as the minimum bactericidal concentration.

Results

Based on the dry weight of the plant, the obtained yield of the essential oil was determined as 0.9% (v/w).

The chromatogram of GC-MS (Figure 1) showing the chemical constituents of the essential oil extracted from air-dried leaves of *O. ramonense* identified 20 compounds comprising 97.8% of the total oil (Table I). The oil was characterized by a large amount of oxygenated monoterpenes (86.9%) followed by monoterpenes hydrocarbons (9.8%). The carvacrol (84.6%), p-cymene (4.3%) and γ -terpinene (3.3%) were the major components. The other compounds such as α -thujene, β -pinene, α -terpinene, cis-sabinene hydrate, terpinen-4-ol, thymol, 5-methoxy-1, 2, 3-trimethylbenzene and caryophyllene, which were ranged from 0.96 to 0.36%. The oil had lower contents of linalool, cis-dihydrocarvone, carvotanacetone, α -phellandrene, geranial, α -humulene, γ -cadinene, caryophyllene oxide and δ -cadinene (less than 0.26% each).

The essential oil clearly showed inhibition activity against all tested bacteria (Table II). The results of disc diffusion method using *O. ramonense* essential oil showed that *S. aureus*, *S. epidermidis* and *E. coli* were the most sensitive clinical isolates with inhibition zones of 28.3, 26.2 and 24.5 mm, respectively followed by *E. aerogenes*, *P. aeruginosa* and *K. pneumoniae* (21.3, 20.7 and 20.2 mm, respectively). While *P. mirabilis* exhibited the smallest inhibition zone (16.7 mm).

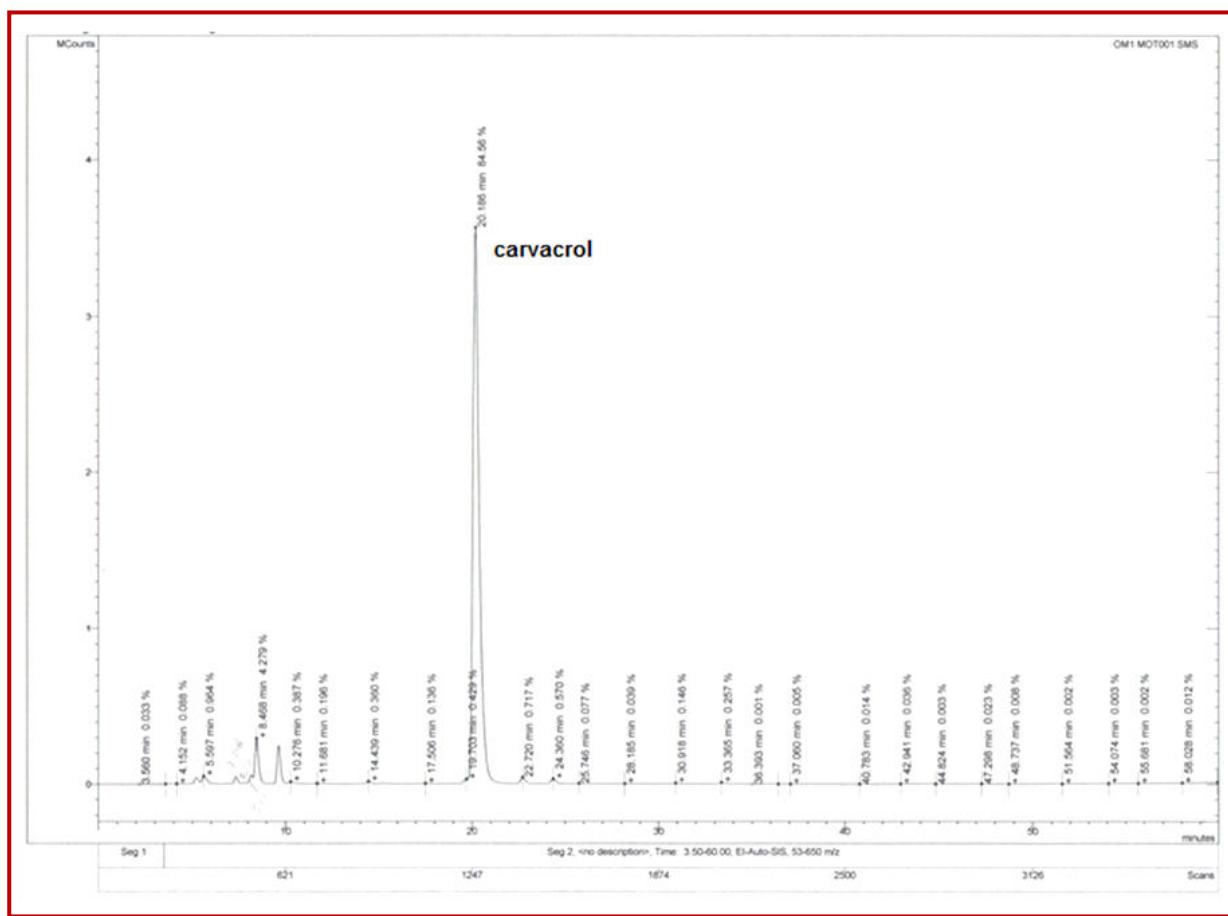


Figure 1: Chromatogram of the essential oil extracted from air-dried leaves of *O. ramonense*

Table I					
Essential oil composition (%) of the air-dried leaves of <i>O. ramonense</i>					
No.	RT	RI	Compound	%Area	Mode of identification
1	5.6	925	α -Thujene	0.96	MS, RI
2	7.3	991	β -Pinene	0.60	MS, RI
3	7.8	1006	α -Phellandren	<0.1%	MS, RI
4	8.2	1017	α -Terpinene	0.60	MS, RI
5	8.5	1025	p-Cymene	4.28	MS, RI
6	9.6	1058	γ -Terpinene	3.30	MS, RI
7	10.3	1076	Cis-Sabinene hydrate	0.39	MS, RI
8	11.7	1113	Linalool	0.20	RI
9	14.4	1181	Terpinen-4-ol	0.36	MS, RI
10	15.3	1201	cis-Dihydrocarvone	0.15	RI
11	17.2	1247	Carvotanacetone	0.14	RI
12	18.3	1273	Geranial	<0.1%	RI
13	19.7	1307	Thymol	0.43	MS, RI
14	20.2	1318	Carvacrol	84.56	MS, RI
15	22.7	1379	5-methoxy-1, 2, 3-trimethylbenzene	0.72	MS, RI
16	24.4	1419	Caryophyllene	0.57	MS, RI
17	25.7	1453	α -Humulene	<0.1%	MS, RI
18	28.2	1514	γ -Cadinene	<0.1%	MS, RI
19	30.9	1585	Caryophyllene oxide	0.15	MS, RI
20	33.4	1651	δ -Cadinene	0.26	MS, RI
			Total identified	97.78	
			Monoterpenes hydrocarbons	9.76	
			Oxygenated monoterpenes	86.94	
			Sesquiterpene hydrocarbons	0.94	
			Oxygenated sesquiterpenes	0.15	

Table II		
Antibacterial activity of <i>O. ramonense</i> EOs using disc diffusion method		
Bacterial species	Inhibition zone (mm)	Ciprofloxacin
<i>Escherichia coli</i>	24.5 ± 0.5	24.3 ± 0.6
<i>Klebsiella pneumoniae</i>	20.2 ± 1.6	20.8 ± 0.8
<i>Enterobacter aerogenes</i>	21.3 ± 0.8	20.5 ± 0.5
<i>Pseudomonas aeruginosa</i>	20.7 ± 0.3	23.3 ± 0.6
<i>Proteus mirabilis</i>	16.7 ± 0.3	19.2 ± 0.28
<i>Staphylococcus aureus</i>	26.2 ± 1.4	22.0 ± 1.7
<i>Staphylococcus epidermidis</i>	28.3 ± 0.6	22.5 ± 0.5
<i>E. coli</i> 25922	22.7 ± 0.6	22.8 ± 0.8
<i>P. aeruginosa</i> 10145	23.8 ± 0.8	24.3 ± 0.6

Each disc contains 3 μ L of Eos; Ciprofloxacin disc contains 5 μ g

The minimum inhibitory concentrations and minimum bactericidal concentrations of *O. ramonense* essential oil for the tested bacteria indicated that all tested bacterial strains were highly susceptible to *O. ramonense* essential oil. *S. aureus*, *S. epidermidis*, *K. pneumoniae* and *E. aerogenes* had the lowest minimum inhibitory concentration values (0.015 μ g/mL) followed by *E. coli* (0.14 μ g/mL; Table III). The lowest susceptible strains to essential oil were *P. mirabilis* (1.2 μ g/mL) and *E. coli* 25922 (11.1 μ g/mL) *P. aeruginosa* and the reference strain *P. aeruginosa* 10145 exhibited similar minimum inhibitory concentration values with 11.1 μ g/mL. In general, the minimum bactericidal concentration and minimum inhibitory concentration values were observed to be equal or close for most of the investigated strains. The exceptions of obtained data for minimum bactericidal concentration were that of *P. mirabilis* and *E. aerogenes* in which the minimum bactericidal concentration values are 3-fold and 9-fold of the minimum inhibitory

Table III		
Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) of <i>O. ramonense</i> EOs		
Bacterial species	MIC (µg/mL)	MBC (µg/mL)
<i>Escherichia coli</i>	0.14	0.14
<i>Klebsiella pneumoniae</i>	0.015	0.015
<i>Enterobacter aerogenes</i>	0.015	0.14
<i>Pseudomonas aeruginosa</i>	11.1	11.1
<i>Proteus mirabilis</i>	1.2	3.7
<i>Staphylococcus aureus</i>	0.015	0.015
<i>Staphylococcus epidermidis</i>	0.015	0.015
<i>E. coli</i> 25922	11.1	11.1
<i>P. aeruginosa</i> 10145	11.1	11.1
MIC and MBC were determined using a concentration ranged from 33.3 to 0.015 µg/mL		

concentration values, respectively.

Discussion

In this study, the GC-MS analysis of the *O. ramonense* essential oil pointed out that oxygenated monoterpenes (86.9%) as the major component with carvacrol as predominate component. Previous analysis of the essential oil from the *O. ramonense* (Danin et al., 1997) led to the identification of most dominated compounds of *O. ramonense* obtained in this study. These were oxygenated monoterpenes although they were represented by α -terpineol (41.5%), terpinen-4-ol (16.8%), cis-sabinene hydrate (13.2%) and eugenol (3.6%). In this study, GC-MS analysis showed a lack of α -terpineol and eugenol while terpinen-4-ol and cis-sabinene hydrate were indicated at low concentrations. Furthermore, the major component monitored in this study carvacrol (84.6%) whereas too lower concentration (0.1%) was reported (Danin et al., 1997).

The chemical composition of essential oil of various *Origanum* species has been determined. Collectively, more than 60 different compounds have been identified. The most frequent compounds identified from *Origanum* species include carvacrol, β -fenchyl alcohol, thymol, and γ -terpinene (Vazirian et al., 2015). For instance, the major compounds of *O. vulgare* essential oil (Teixeira et al., 2013) include carvacrol, thymol, γ -terpinene, and p-cymene while the main components of *O. onites* essential oil (Ozdemir et al., 2017) include thymol, γ -terpinene, p-cymene, and carvacrol.

Three white-flowered biotypes of *O. vulgare* studied were growing wild in different locations of Italy (De Falco et al., 2014). Their essential oil was rich with

either carvacrol or thymol whereas phenols content was the lowest among them. Factors such as genetic makeup, harvesting seasons, geographical distribution and the extraction methods collectively might affect the chemical composition of essential oil (Ozdemir et al., 2017). Chemical analysis of the essential oil from these plants showed a high variability in the essential oil composition and content, indicating that various compounds may account for the different extents of the antimicrobial activity of plants

In this study, our results revealed the potential broad spectrum antibacterial activity of *O. ramonense* essential oil. This potent activity could be because of the presence of its main component carvacrol. The antibacterial potency of carvacrol and its isomer thymol, has been reported with extremely low minimum inhibitory concentration and minimum bactericidal concentration values (Qaralleh et al., 2009; Althunibat et al., 2016). Carvacrol is considered as one of the most bactericidal essential oil components against both Gram positive and Gram negative bacteria (Hammer et al., 1999; Nieto, 2017). Mihajilov-Krstev et al., showed that essential oil (*Satureja hortensis*) rich in carvacrol (67%) and γ -terpinene (15%) are strong bacteriostatic and bactericidal agent against both Gram positive and Gram negative bacteria with low minimum inhibitory concentration and minimum bactericidal concentration values (0.025–0.78 µL/mL) (Mihajilov-Krstev et al., 2010). Other compounds such as p-cymene and gamma terpinene are also known for their antibacterial activity. Nevertheless, the antibacterial mechanism at the cellular and molecular level of oregano essential oil against several bacterial strains were previously reported. For instance, the treatment of *E. coli* O157:H7 with *O. vulgare* essential oil caused an increment in the cell membrane permeability and leakage of cell contents (Burt and Reinders 2003). *Pseudomonas aeruginosa* and *S. aureus* treated with *O. compactum* essential oil suffered from membrane damage (Bouhdid et al., 2009). In addition, oregano EOs and carvacrol have been shown to down-regulate the expression of virulence genes in enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 (Mith et al., 2015). The essential oil concentration is largely related issue to species, season of gathering the plant, geographical place, the plant part that is used, and the method of oil extraction (Sakkas and Papadopoulou, 2017).

However, several essential oils extracts from different medicinal plants were previously reported with potent antibacterial activity against urinary tract infection isolates such as *E. coli*, *K. pneumonia*, *S. aureus*, *Streptococcus agalactiae*, *S. epidermidis*, *Enterococcus faecalis*, *S. saprophyticus*, *Enterobacter spp*, *Shigella spp*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Morganella morganii* (Amalaradjou and Venkitanarayanan, 2011; Beydokthi et al., 2017; Kumar et al., 2012; Saeed et al.,

2016; Susanti et al., 2013). In contrast, essential oil extract of *Oregano* is not traditionally used to treat urinary tract infection and information about *Oregano* utilization in treating or controlling urinary tract infection in the literature are rare. Therefore, the results of this study clearly show that *O. ramonense* essential oil can be used to treat the urinary tract infection patients infected with such urinary tract infection causing agents.

Conclusion

The bacteriostatic and bactericidal effect at concentration as low as 0.015 µg/mL indicated the potent antibacterial activity of *O. ramonense* essential.

Conflict of Interest

There is no conflict of interest.

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