Avicenna Journal of Clinical Microbiology and Infection
doi:10.15171/ajcmi.2019.06 2019 February;6(1):26-30 http://ajcmi.umsha.ac.ir

Original Article

In Vitro Antibacterial Activity of *Origanum syriacum* L. Essential Oils Against Gram-Negative Bacteria

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Received: 8 Oct. 2018
Accepted: 23 Jan. 2019
ePublished: 28 Feb. 2019

Abstract

Background: This study aimed to determine the chemical composition of *Origanum syriacum* L., as well as to assess the antimicrobial activity of *O. syriacum* essential oil and its constituents.

Methods: To this end, *O. syriacum* plants were collected from their native growing locations in western and coastal governorates of Syria. Next, the composition of the essential oil from every station was determined by gas chromatography (GC) and then by high-performance liquid chromatography (HPLC) to estimate the number and the quantity of all components. Preparative-HPLC was used to isolate the essential oil components. Then, the identified constituents of the essential oils were confirmed utilizing GC-mass spectrometry. Microdilution broth susceptibility assay was applied and the first well without turbidity was considered as the minimum inhibitory concentration.

Results: The results showed that ß-myrcene (21.93%), carvacrol (19.20%), anisaldehyde (7.57%), thymol (7.40%), γ–terpinene (5.27%), and sabinene (4.43%) were the main components of bulk essential oils. Similarly, only minor qualitative and quantitative variation was found between locations. The antibacterial activity of bulk essential oil and its components was evaluated against gram-negative local isolates of *Escherichia coli* O157, *Salmonella enterica*, *Klebsiella pneumoniae*, *Yersinia enterocolitica* O9, *Brucella melitensis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. Although the bulk essential oil inhibits all the bacteria except for *E. coli* O157 at the highest tested concentration (48 µL/mL), the essential oil components differ in their antibacterial activity.

Conclusions: Overall, thymol and carvacrol represented the most antibacterial activity compared to the other substances.

Keywords: Essential oil, Minimum inhibitory concentration, *Origanum syriacum,* Gram-negative bacteria

Background

Origanum is a flowering herbaceous perennial plant in the family *Lamiaceae* that contains more than 40 species. It is also native to the Mediterranean and southeastern Asia and is found in open or mountainous habitats. This plant has strong aromatic leaves and abundant tubular flowers with long-lasting colored bracts (1). The genus *Origanum* is represented by one species (*Origanum syriacum*) which is native and widely cultivated in Syria (2,3). In addition, this aromatic plant is commonly used by Syrian community as spices, herbal tea, and condiments, as well as in traditional medicine and it has many vernacular names such as Mardakosh or Bardakosh or Zatar AL Khalil (4). The importance of such plants is increasing worldwide as conservation alternatives. Therefore, the food industry must lower the salt and sugar contents by replacing them with other safe natural preservative substances, especially in regularly consumed foods (5, 6). Although the *Origanum* species exhibits high antibacterial and antifungal activity, it is safe to be taken orally by mammalians at a dose LD_{50} 2790 mg, LD_{50} 980 mg, and LD_{50} 810 mg for the essential oil, thymol, and carvacrol, respectively (7). According to the Food and Agriculture Organization (FAO)/World Health Organization (WHO), *Origanum* is regarded as one of the food flavorings and thus it is safe based on the current estimated levels of intake (8). Further, *O. syriacum* essential oil is known as therapy adjuvants in many diseases, especially for the expectorant, spasmolytic, antioxidant, antimicrobial, choleretic, and eupeptic properties (1). Carvacrol and thymol are the main phenolic compounds responsible for most of the therapeutic properties (1).

According to FAO/WHO of the United Nations, many researchers confirm the antifungal activity of *O. vulgare* L. it was summarized by (7). For example, Santos et al noticed that 2.0 to 2.5 µL/L of air in a fumigation chamber inhibited the mycelial growth and eradicated the spores of *Aspergillus flavus, A. Niger*, and *A. ochraceus* (the fungal pests of the stored products). Sporulation and aflatoxin

production was also inhibited in some toxigenic strains of *Aspergillus* cultured on ground *O. vulgare* (1.5 g) and sterile water for 30 days (8). In addition to its anti-fungal action, *O. vulgare* and *O. majorana* essential oils have a strong anti-microbial activity against a wide number of bacteria including *Escherichia coli, Klebsiella pneumoniae, Salmonella enterica*, and *Staphylococcus aureus* (9).

Rhayour et al simultaneously studied the mechanism of the antimicrobial activity of *O. compactum* essential oils with thymol and eugenol. They further used *E. coli* and *B. subtilis* as Gram-negative and Gram-positive bacterial models, respectively. Based on their findings, the essential oils and their major components were capable of inducing the cell lysis. Moreover, bacteria lysis was observed by the release of substances absorbed at 260 nm. For *E. coli*, the results were similar to those obtained with polymyxin B. Finally, scanning electronic microscope observations revealed that both the cell wall and the membrane of the treated bacteria damaged significantly (10).

A sample of *O. vulgare* essential oil was administered to *S. aureus* and *P. aeruginosa*, followed by studying their effect on potassium and phosphate leakage. The results confirmed the antimicrobial effects of the *O. vulgare* essential oil by increasing the membrane permeability, thus this mechanism was considered as a major factor of antimicrobial activity (11).

Considering the above-mentioned discussions, this investigation aimed to determine the chemical composition of *O. syriacum* and to evaluate the antimicrobial activity of its essential oil and constituents.

Materials and Methods

Bacterial Strains

The local gram-negative strains of *E. coli* O157, *S. enterica, K. pneumoniae, Yersinia enterocolitica* O9, *Brucella melitensis, Proteus mirabilis,* and *P. aeruginosa* were isolated from incoming food and patients' samples and transferred to the Microbiology Labs in the Atomic Energy Commission of Syria. The bacterial identification was achieved using selective culture media, which were purchased from Fluka, Biolife, and Himedia, and then it was confirmed by polymerase chain reaction technique using specific primers for each strain. The isolates were freshly grown and 1×10^8 CFU/mL was standardized in LB broth.

Collection and Preparation of Plant Material

The branches (leaves and buds) of *O. syriacum* were collected during the flowering season, followed by cleaning the samples from any strange plants, dust, or any other contaminants. Then, the collected plants were air-dried and cut into pieces.

Information about the plant collection locations and the percentages of the essential oils in the collected dried samples are presented in Table 1.

Isolation of Essential Oil

The isolation of essential oils was acquired applying water steam distillation device (Clevenger-type apparatus, Heidelberg, Germany) attached to a condenser and cold water recycler (hydrodistillation technique) according to the European Pharmacopoeia Method (12). Then, double distilled water was added (1:10 m/v) and each sample was distilled for three hours. Next, the supernatant essential oil was filtered through anhydrous Na_2SO_4 to dry the yield essential oil. Finally, the essential oil was collected in tightened vials and stored in a refrigerator under nitrogen.

Identification and Isolation of Essential Oil Components

The composition of the essential oil from every station was determined by gas chromatography (GC) and then by high-performance liquid chromatography (HPLC) to estimate the number and the quantity of all components. The essential oils were then analyzed using an Agilent (6890N) GC system (GmbH & Co. KG; USA). The employed capillary column was DB-5 (30 m×0.25 mm i.d., 0.25 µm film thickness) with helium as the carrier gas at 1 mL/min. In addition, the initial temperature of the column was 45°C (held 2 minutes) and then heated to 175°C at a rate of 3°C/min (held 5 minutes), followed by heated to 275°C at a rate of 4°C/min (held 10 minutes). The temperature of the injector and flame ionization detection was set at 275°C and 300°C, respectively.

Likewise, the component fractions from the isolated essential oil were separated by a semi-preparative HPLC instrument JASCO-LC-1500 (JASCO Benelux B.V.; Japan) equipped with a UV/VIS detector and the ODS C18 semi-preparative column.

The operation conditions included tetrahydrofuran/acetonitrile/H_2O as a mobile phase, a flow rate of 1.1 mL/min, an injected sample volume of 1500 µL, and the analysis time of about 65 minutes. Further, the used wavelength was 254 nm and the retention times (RT) of some individual constituents were compared with those of available authentic samples in order to check the credibility of the determinations.

Table 1. Characteristics of Collection Locations

Locations No.	Location Name	Altitude (m)	Maximum Temperature a Year (°C)	Average Precipitation (mm/year)	EO%
1	Idlib (Has)	446	42.5	436	2.24
2	Idlib Kafr (Nobol)	446	42.5	436	2.31
3	Latakia (Kasab)	1521	27.1	1521	2.1

Similarly, the constituents of the essential oils were confirmed employing GC-mass spectrometry (MS). The GC-MS analysis was performed using an Agilent GC-MS model GC-6890 (Wilmington, DE 19808-1610 USA), the mass selective detector 5973 inert. The capillary column was DB-35 (30x0.2 mm, film thickness 0.25 µm) as well. The operating conditions were the carrier gas, helium with a flow rate of 1 mL/min, as well as the injection volume of 1 µL of the essential oil, and ionization potential of 70ev. The initial temperature of the column was 50°C (held 2 minutes) and then, it was heated to 170°C at a rate of 2°C/min (held 7 minutes), followed by heating to 250°C at a rate of 4°C/min (held 10 minutes). The identification of the components of the essential oil was based on RT. Individual components were identified by the comparison of both mass spectra and their GC retention data. Other identifications were also made by comparing the mass spectra with those in the data system libraries.

Determination of Minimum Inhibitory Concentrations (MICs)

Microdilution broth susceptibility assay was used (13) and three replicates of the serial dilutions (0.5-50 µL/mL) of the essential oils (the bulk oil was obtained by combining three oil samples) and their components were prepared in LB broth medium in 96-well microtiter plates. Then, 100 µL of freshly grown bacteria (1×10^8 CFU/mL), standardized in LB broth, were added to each well.

Positive and negative control was performed with the same conditions but without adding the essential oils or the bacteria, respectively. The plate was incubated by shaking for 24 hours at 37°C. The first well without turbidity was considered as the MIC.

Results and Discussion

The plant samples were collected from 2 governorates and three locations. Table 1 represents collection site characteristics and the percentage of the essential oils in the dried samples. In addition, Table 2 shows the composition of *O. syriacum* essential oil for each location that identifies 15 compounds representing 92.57±1.46% of the crude oil.

The main constituents of *O. syriacum* essential oils were ß–myrcene (21.93%), carvacrol (19.20%), anisaldehyde (7.57%), thymol (7.40%), γ–terpinene (5.27%), and sabinene (4.43%), which is compatible with the results of other studies (14--16).

Daouk et al studied the composition of *O. syriacum* (Lebanese strain) for its thymol and carvacrol contents (17). Based on their results, these two compounds constituted the major components of the essential oil and were presented in equal proportions of 30% in the essential oil isolated from the leaves and shoot tips of the *O. syriacum* plant during the pre-flowering stage. The percentage of carvacrol in the essential oil increased to 62% after flowering and maturation while the concentration of thymol decreased to 14%.

Further, Burt reviewed and summarized the composition of *O. vulgare* essential oils as the carvacrol available in the approximate range trace-80%, thymol trace-64%, and p-cymene trace-52% (18).

Loizzo et al also reported that *O. syriacum* essential oil contains 36 compounds representing 90.6% of the total oil (19). The most abundant components were thymol (24.7%), carvacrol (17.6%), γ-terpinene (12.6%), p-cymene (8.7%), 2-isopropyl-1-methoxy-4-methylbenzene (7.9%), and α-terpinene (2.5%). According to the previous report, *O. syriacum* var. *bevanii* growing in Turkey contained carvacrol (64.1%) and p-cymene (12.3%) as the major components (20). On the other hand, the bulk oil of *O. syriacum* from Egypt only contained carvacrol (76.7%) as the main constituent (21).

Based on the composition of *O. syriacum* crude oil, it was revealed that it is rich in ß–myrcene and carvacrol for all collection sites with minor qualitative and

Table 2. The Composition of *Origanum syriacum* Essential Oils

Compound	RT (min)	Location 1	Location 2	Location 3	Average %
α-Pinene	4.3	2.1	1.9	1.4	1.80
Sabinene	5.6	6.3	4.3	2.7	4.43
ß–Myrcene	7.8	23.1	24.9	17.8	21.93
Limonene	10.3	1.3	1.2	0.4	0.97
p-Cymene	12.4	3.6	5.2	14.2	7.67
1,8-Cineole	15.6	4.4	3.3	1.3	3.00
γ–Terpinene	16.7	5.3	4.1	6.4	5.27
Linalool	17.4	1.2	1.3	2.2	1.57
Terpinen-4-ol	18.2	1.1	0.8	1.5	1.13
Thymol	19.7	7.5	5.5	9.2	7.40
Neral	21.3	1.6	2.2	1.4	1.73
Anis aldehyde	22.9	5.9	7.6	9.2	7.57
Cis-carvyle acetate	23.8	4.8	6.3	2.7	4.60
Carvacrol	25.3	19.6	17.9	20.1	19.20
ß-Caryophyllene	27.6	4.3	4.9	3.7	4.30
Total identified		92.1	91.4	94.2	

quantitative variations between the locations. This is in line with the findings of Azizi et al which indicated that the composition of the essential oil of *O. vulgare* populations was independent of cultivation conditions (22). However, Mockute et al, Kumar et al, and many other researchers concluded that the chemical composition of the essential oil of *O. vulgare* L. is influenced by the geographical location of collection sites due to climatic and other ecological conditioning factors (23,24).

In this study, the bulk oil was obtained by combining equal amounts of three oils. The bulk oils and their constituents were then tested for their minimum inhibitory concentrations (MIC) against the local isolates of gram-negative bacteria (i.e., *E. coli* O157, *S. enterica, K. pneumoniae, Y. enterocolitica* O9, *B. melitensis, P. mirabilis,* and *P. aeruginosa*). The MIC values of the essential oil and its constituents are presented in Table 3. The bulk oil showed an antibacterial activity in the concentration of 0.75-48 µL/mL against *Y. enterocolitica* O9, *B. melitensis, P. mirabilis, P. aeruginosa*, and *S. enterica,* but it was not effective against *E. coli* O157. Moreover, thymol, neral, and carvacrol represented antibacterial activity against all tested bacterial strains in the microdilution method. Additionally, thymol demonstrated an antibacterial activity in the concentration of 0.75-48 µL/mL, which was less than MIC values against the most tested strains, thus it can be considered as the most active component. In contrast, β-myrcene, limonene, and p-cymene were inactive against all tested strains (i.e., *E. coli* O157, *S. enterica, K. pneumoniae, Y. enterocolitica* O9, *B. melitensis, P. mirabilis,* and *P. aeruginosa*).

The extracted essential oil of *O. syriacum* was evaluated by Daouk for its antifungal activity against *Penicillium* species, *A. niger*, and *Fusarium oxysporum* and the results indicated a strong inhibitory effect against all tested fungi. The MIC of the essential oil was found to be 0.1 µL/mL for all tested fungal strains (17).

Comparing the MIC values for thymol and carvacrol, we found that thymol is more effective than carvacrol for the most tested strains while in other studies the influence was similar in both of these components (18,25,26), which can be attributed to the resistance features of the local strains.

Likewise, the MIC values for bacterial strains, which were sensitive to the essential oil of *O. vulgare* sp. *vulgare*, included 15.62–125 µL/mL according to Fleisher et al (21). Whereas Aslim and Yucel found that MIC values of 7.8–800 µg/mL for *O. minutiflorum* essential oil on 21 isolates of ciprofloxacin-resistant *Campylobacter* spp (27). In addition, Busatta et al reported their MIC results for marjoram essential oil on bacteria as 0.920 mg/mL, 0.069 mg/mL, 2.300 mg/mL, 0.920 mg/mL, 0.920 mg/mL, 0.920 mg/mL, 2.300 mg/mL, 0.782 mg/mL, (0.782, and 2.300 mg/mL for *Aeromonas* sp, *Bacillus subtilis, Enterococcus faecalis, E. coli, K. pneumoniae, S. choleraesuis, Serratia* sp., *Shigella flexneri, Staphylococcus aureus,* and *Streptococcus mutans,* respectively. Busatta et al also mentioned that MIC average values were 1.127 mg/mL and 1.263 mg/mL for gram-negative and gram-positive bacteria, respectively (28).

Conclusions

Overall, the results of this study suggest the possibility of using the essential oil of the Syrian *O. syriacum* L. and some of its components as medical drugs or natural food preservatives. Nevertheless, more investigation is required to explore the possibility of introducing this effective oil to many medical therapies and food industries.

Table 3. The MIC (µL/mL) Values for Essential Oil and Their Constituents

Essential Oil and Constituents (µL/mL)	*E. coli* O157	*Y. entrocolitica O9*	*B. melitensis*	*P. mirabilis*	*P. aeruginosa*	*S. enterica*	*K. pneumoniae*
EO bulk extract	NIE	1.5	3.0	1.5	1.5	0.75	48
α-Pinene	NIE	NIE	NIE	NIE	NIE	NIE	48
Sabinene	48	48	48	48	48	48	NIE
ß-Myrcene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
Limonene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
p-Cymene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
1,8-Cineole	48	48	NIE	48	48	48	48
γ-Terpinene	NIE	NIE	NIE	NIE	NIE	NIE	48
Linalool	24	48	NIE	48	NIE	48	NIE
Terpinen-4-ol	NIE	48	12.5	6.25	25	6.0	12.5
Thymol	3.0	0.75	3.0	1.5	6.0	0.75	1.5
Neral	48	12.0	48	48	48	48	48
Anis aldehyde	48	48	48	48	48	48	48
Cis-carvyle acetate	48	48	NIE	NIE	NIE	NIE	NIE
Carvacrol	6.0	48	6.0	1.5	6.0	6.0	6.0
ß-Caryophyllene	NIE	NIE	NIE	NIE	NIE	48	NIE

Ethical Approval

The disclaimer implies that ethical principles have been reviewed in relation to the proposed work and no ethical issues have been found to apply to the research proposal.

Conflict of Interest Disclosures

No competing interest was declared by any of the authors.

Financial Disclosure

The author has no financial interests related to the material in the manuscript

Acknowledgment

The authors would like to thank the General Director of the Atomic Energy Commission of Syria and the Head of the Department of Molecular Biology and Biotechnology for their support.

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