JOURNAL OF MEDICINAL FOOD
J Med Food 16 (12) 2013, 1115–1120

DOI: 10.1089/jmf.2013.0079

Essential Oil Composition and Antibacterial Activity of *Origanum vulgare* subsp. *glandulosum* Desf. at Different Phenological Stages

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ABSTRACT Variation in the quantity and quality of the essential oil (EO) of wild population of *Origanum vulgare* at different phenological stages, including vegetative, late vegetative, and flowering set, is reported. The oils of air-dried samples were obtained by hydrodistillation. The yield of oils (w/w%) at different stages were in the order of late vegetative (2.0%), early vegetative (1.7%), and flowering (0.6%) set. The oils were analyzed by gas chromatography (GC) and GC–mass spectrometry (GC-MS). In total, 36, 33, and 16 components were identified and quantified in vegetative, late vegetative, and flowering set, representing 94.47%, 95.91%, and 99.62% of the oil, respectively. Carvacrol was the major compound in all samples. The ranges of major constituents were as follows: carvacrol (61.08–83.37%), *p*-cymene (3.02–9.87%), and γ-terpinene (4.13–6.34%). Antibacterial activity of the oils was tested against three Gram-positive and two Gram-negative bacteria by the disc diffusion method and determining their diameter of inhibition and the minimum inhibitory concentration (MIC) values. The inhibition zones and MIC values for bacterial strains, which were sensitive to the EO of *O. vulgare* subsp. *glandulosum*, were in the range of 9–36 mm and 125–600 μg/mL, respectively. The oils of various phenological stages showed high activity against all tested bacteria, of which *Bacillus subtilis* was the most sensitive and resistant strain, respectively. Thus, they represent an inexpensive source of natural antibacterial substances that exhibited potential for use in pathogenic systems.

KEY WORDS: • ***antibacterial activity*** • ***essential oils*** • ***GC-MS analysis*** • ***growth stages*** • **Origanum vulgare *subsp.* glandulosum**

INTRODUCTION

THE GENUS *Origanum* L., (Lamiaceae), comprises 38 species of annual, perennial, and shrubby herbs, most of which are native to or restricted to the eastern part of the Mediterranean area, Europe, Asia, and North Africa.[1] The antioxidant and other biological properties of the *Origanum* essential oils (EO) and extracts have recently been of great interest in both academia and food industries because of their antioxidant and antimicrobial potentials.

Oregano is one of the most commonly known culinary herbs worldwide for cooking purposes. The dried herbs are used in many processed foods such as alcohol beverages, meat products, snack foods, and milk products. Some of the *Origanum* spp. are also used as a fragrance component in soaps, detergents, perfumes, cosmetics, flavorings, and pharmaceuticals.[2] Oregano oil has antibacterial, antifungal, antiparasitic, antimicrobial, and antioxidant properties. Even though the EOs and the constituents of many *Origanum* species have been studied,[3,4] only few reports on the antimicrobial and antioxidant activities of the *Origanum* EOs are available to date.[5–7]

According to the Flora of Tunisia, the genus *Origanum* is constituted by three species. Among them, *Origanum vulgare* subsp. *glandulosum* (Desf.) Ietswaart, synonymous *O. glandulosum* Desf., is an endemic spontaneous plant growing in North Africa (Algeria and Tunisia).[8] In Tunisia, *O. vulgare* subsp. *glandulosum* is an aromatic shrub called ''zaâter el Mlouk'' (thyme of kings), which is mostly used as a medicinal plant against whooping cough, cough, fever, and bronchitis.[9]

Bejaoui *et al.*[10] showed that this species is a rich source of phenolic monoterpenes and carvacrol. Considering that carvacrol-rich EOs are gaining increasing importance for their considerable antimicrobial and antioxidant activity,[11] the present study reports the EO composition of this species at different developmental stages and its coherence with antibacterial activity. These results can be used to investigate the optimal harvesting time of this plant for relevant industries.

Manuscript received 14 March 2013. Revision accepted 2 September 2013.

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MATERIALS AND METHODS

Plant material

The oregano plants used for this study were collected from the Sidi Nssir Mountain (35°27′N, 9°33′E) located in the North East of Tunisia. Botanical identification of this species was carried out by Prof. Mohamed Boussaid, biologist (National Institute of Applied Science and Technology, Tunisia). Voucher specimens (number: OG5) were deposited at the herbarium of INSAT.

Isolation of EOs

The EOs were obtained by hydrodistillation using a Clevenger-type apparatus for 3 h from 50 to 100 g of air-dried leaves of each sample. Oil yields were then estimated on the basis of the dry weight of plant material. Oils were recovered directly, from above the distillate, and stored in dark vials at 4°C.

EO identification

Gas chromatography (GC) analyses were performed using an Agilent 6890 N gas chromatograph equipped with a flame ionization detector and an electronic pressure control injector. Nonpolar HP-5 MS columns (30 m × 0.25 mm, 0.25 μM film phenyl methyl siloxane) were used. The carrier gas was helium with a flow rate of 1 mL/min. The split ratio was 50:1. All analyses were performed using the following temperature ramp: oven kept isothermally at 50°C for 1 min increased from 50°C to 250°C at the rate of 8°C/min and then kept at 250°C for 10 min. Injector and detector temperatures were held at 220°C and 280°C, respectively.

The EOs were analyzed by gas chromatography–mass spectrometry (GC-MS) using a HP 5975C mass spectrometer (Agilent Technologies) with electron impact ionization (70 eV). A HP-5MS capillary column (30 m × 250 μM coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25 μM film thickness) was used. Oven temperature was programmed to rise from 60°C to 220°C at a rate of 4°C/min; transfer line temperature was 230°C. The carrier gas was He, with a flow rate of 0.8 mL/min and a split ratio of 50:1. The scan time and mass range were 1 s and 50–550 *m*/*z*, respectively.[12]

The identification of oil components was assigned by comparison of their retention indices relative to (C8–C22) *n*-alkanes with those of literature or with those of authentic compounds available in the authors' laboratory. Further identification was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC-MS data system and other published mass spectra.[13] Determination of the percentage composition was based on peak area normalization without using correction factors.

Antimicrobial activity

Microbial strains. EOs from different growth stages were individually tested against a panel of microorganisms, including Gram-positive bacteria and Gram-negative bacteria. Microorganisms were provided from the culture collection of the Laboratory of Natural Substances, at the National Institute of Research and Physico-chemical Analysis. The Gram-positive bacteria were *Staphylococus aureus* (strain ATCC 6538) and *Bacillus subtilis* (environmental strain) and the Gram-negative bacteria were *Escherichia coli* (strain ATCC 8739), *Salmonella typhimurium* (strain ATCC 14028), and *Pseudomonas aeruginosa* (strain NCTC 10418).

Antimicrobial activity assays

The antimicrobial activity of oils was determined through the agar disc diffusion and the microdilution broth susceptibility assay. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined. All tests were performed in triplicate.

Disc diffusion method

A suspension of the tested microorganisms was spread on the appropriate solid media plates and incubated overnight at 37°C. After 1 day, 4–5 loops of pure colonies were transferred to a saline solution in a test tube for each bacterial strain and adjusted to the 0.5 McFarland turbidity standard ($\sim 10^8$ cells/mL). Sterile cotton dipped into the bacterial suspension and the agar plates were streaked three times, each time turning the plate at a 60° angle and finally rubbing the swab through the edge of the plate. Sterile paper discs (Glass Microfiber filters, Whatman; 6 mm diameter) were placed onto inoculated plates and impregnated with oil solutions diluted at 1:16 in dimethyl sulfoxide (DMSO). Ampicillin (10 μg/disc) was used as positive control for all strains except *P. aeruginosa* for which gentamicine (15 μg/disc) was used. This control allows examining the microorganism density.[14] Inoculated plates with discs were placed in a 37°C incubator. After 24 h of incubation, the results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The test was run in triplicate.

Determination of MIC and MBC

The MIC is determined by the microdilution method.[15] The inoculum of the microorganisms was prepared using 24-h cultures and suspensions were adjusted to the 5.0 McFarland turbidity standard and further diluted with a sterile physiological saline solution to achieve $\sim 10^6$ CFU/mL. Stock solutions of the EOs were prepared in DMSO and then serial dilutions of the EO were made in a concentration range from 250 to 3.90 μg/mL. A sterile 96-well microplate was used for each strain and prepared by dispersing in each well, 100 μL of the oil dilution added to 5 μL of the inoculum and 95 μL of the sterile MH broth. The final volume in each well was 200 μL. These wells are duplicated for each EO. A positive control (containing 5 μL inoculum and 195 μL MH broth) and negative control (containing 100 μL of EO dissolved in DMSO, 100 μL of MH broth without

inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 37°C for 24 h. After incubation, 10 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/mL distilled H_2O) was added to each well and the plates were incubated for a further 30 min and the MIC was determined as the lowest concentration of test sample before purple formazan crystals were observed.

Referring to results of the MIC assay, the MBC was determined by dispersing 100 μL from each well remaining yellow (no purple formazan crystals were observed) on nutrient agar plates. After incubation at 37°C for 24 h, the bactericidal concentration corresponds to the plate concentration in which no colonies were grown. The results obtained from antibacterial bioassays are expressed as mean ± standard error of the mean.

Statistical analysis

The results obtained from antibacterial bioassays are expressed as mean ± standard error of the mean. Compound variation among growth stages was assessed by a variance analysis performed on constituents with percentage exceeding 0.01% and 0.1% according to ANOVA procedure.[16] Significance of the *F*-test was estimated at $P < .01$ and $P < .001$. A Duncan multiple range test at $P < .05$ was used to compare averages of constituents among the three developmental stages. Statistical analyses were executed after arc sinus square root transformation of percentages.

RESULTS AND DISCUSSION

Effect of growth stage on chemical components of the EO

The yields of EO of *O. vulgare* subsp. *glandulosum* aerial parts vary during the three growth stages. Changes were observed among the different growth stages for EO yield (w/w) as calculated on the basis of dry matter weight. During the early vegetative stage (EVS), the EO yield is of 1.7%. At the late vegetative stage (LVS), the EO yield reached 2%. At the flowering stage (FS), the EO yields decreased significantly to 0.6%. These results are in disagreement with most of the previous works, which reported that the FS is characterized by the highest EO yield. In fact, Sellami *et al.*[17] reported that the highest EO content of *O. majorana* plants occurred at FS. Our results clearly indicate that harvesting time should be carefully selected to ensure maximum yield of EO.

The oils were obtained by hydrodistillation of air-dried samples and quantified in the subsequent stages, respectively (Table 1). In total, 44 components of the oils have been identified. Percentages of β-myrcene, *p*-cymene, γ-terpinene, sabinene hydrate, borneol, β-caryophyllene, carvacrol, β-bisabolene, and α-cadinol varied significantly among the three developmental stages (Table 1). Thirty-six components accounting for 94.47% of the total oil were identified in the EVS. The major constituents of this oil were carvacrol (61.08%), *p*-cymene (9.87%), γ-terpinen (6.34%), and borneol (2.38%). In the oil obtained from the LVS, 33 constituents representing 95.91% of the total composition

Table 1. Chemical Composition of *Origanum vulgare* subsp. *glandulosum* Essential Oil During Three Developmental Stages

			Sample			
Constituent		*RI*	*EVS*	*LVS*	*FS*	F-*test*
1	α-Thujene	924	1.04	0.8	0.35	ns
2	α-Pinene	940	0.65	0.27	0.16	ns
3	Camphene	953	0.44	–	–	ns
4	Sabinene	979	0.04	–	–	ns
5	β-Pinene	983	0.31	–	–	ns
6	β-Myrcene	992	–[b]	1[a]	0.72[a]	*
7	3-Octanol	995	0.12	–	0.16	ns
8	α-Phellandrene	1006	0.26	0.21	–	ns
9	3-Carene	1009	0.09	0.09	–	ns
10	α-Terpinene	1017	1.37	1.33	0.88	ns
11	*p*-Cymene	1026	9.87[a]	5.4[b]	3.02[c]	**
12	β-Phellandrene	1030	–	0.4	–	ns
13	Trans-α-ocimene	1035	1.39	1.15	1.19	ns
14	β-Ocimene	1040	0.07	0.07	–	ns
15	γ-Terpinene	1059	6.34[a]	4.25[b]	4.13[b]	*
16	*Cis*-sabinene hydrate	1072	0.81	–	–	ns
17	Sabinene hydrate	1086	0.16[b]	1.32[a]	0.65[ab]	*
18	α-Terpinolene	1088	0.19	0.15	–	ns
19	α-Campholene aldehyde	1125	0.08	0.15	–	ns
20	Borneol	1164	2.38[a]	1.38[b]	0.85[b]	*
21	Terpinen-4-ol	1181	0.95	1.18	0.94	ns
22	Carvone	1240	0.09	0.21	–	ns
23	Carvacrol methyl ether	1142	0.14	–	–	ns
24	Thymol	1293	0.21	–	–	ns
25	Carvacrol	1302	61.08[c]	67.93[b]	83.37[a]	**
26	Eugenol	1353	0.04	–	–	ns
27	Carvacryl acetate	1367	0.52	0.47	1.08	ns
28	β-Caryophyllene	1421	1.62[b]	2.5[a]	1.09[b]	*
29	Aromadendrene	1437	0.17	0.16	–	ns
30	α-Humulene	1445	–	0.12	–	ns
31	Germacrene D	1477	0.17	0.09	–	ns
32	α-Amorphene	1487	0.22	0.19	–	ns
33	Bicyclogermacrene	1498	0.38	0.29	–	ns
34	β-Cubebene	1507	–	0.05	–	ns
35	β-Bisabolene	1510	1.64[a]	1.34[a]	–[b]	*
36	γ-Cadinene	1518	–	0.27	–	ns
37	δ-Cadinene	1523	–	0.11	0.63	ns
38	Trans-nerodiol	1568	–	–	0.4	ns
39	Spathulenol	1581	0.36	0.23	–	ns
40	Caryophyllene oxide	1585	0.82	0.43	–	ns
41	β-Eudesmol	1643	0.06	–	–	ns
42	α-Cadinol	1646	–[b]	1.99[a]	–[b]	*
43	δ-Muurolene	1685	0.06	–	–	ns
44	Lanceol	1770	0.33	0.38	–	ns
Total			94.47	95.91	99.62	
Chemical classes						
	Monoterpene hydrocarbons		22.06	15.02	10.45	
	Oxygenated monoterpenes		66.34	72.49	86.89	
	Sesquiterpene hydrocarbons		4.26	5.12	1.72	
	Oxygenated sesquiterpenes		1.57	3.03	0.4	
	Others		0.24	0.15	0.16	

F-test of the variance analysis (F44/9 degrees of freedom) is not significant (ns), highly significant (**$P < .001$), or significant (*$P < .01$).

[abc]Values followed by the same letter are not significantly different (Duncan's multiple range test at $P > .05$).

RI, retention index in a HP-5 MS capillary column; EVS, early vegetative stage; LVS, late vegetative stage; FS, flowering stage.

Table 2. Antibacterial Activity of Essential Oil Against Selected Bacterial Strains

Bacterial strain	Inhibition zone (mm)			
	CON+	EVS (d 1/16)	LVS (d 1/16)	FS (d 1/16)
Escherichia coli ATCC 8739 G(–)	14±0.5	22±2.0	18±0.5	23±2.6
Salmonella typhimurium ATCC 14028 G(–)	15±0.5	20±0.0	15±0.5	22.5±1.8
Ampicillin-resistant *Pseudomonas aeruginosa* (NCTC 10418) G(–)	30±0.5	9±0.5	9±1.1	9±0.2
Staphylococus aureus ATCC 6538 G(+)	30±0.0	19±1.0	15±0.5	19±1.0
Bacillus subtilis G(+)	40±1.1	36±1.5	21±1.0	26±2.0

Values are expressed as mean±standard deviation of inhibition zone diameter (mm) for different growth stages of EO against selected microbial strains. Antibiotic: ampicillin (*E. coli*, *S. typhimurium*, *S. aureus*, *B. subtilis*) and gentamicin (*P. aeruginosa*).

CON+, positive control; G(–), Gram-negative; G(+) Gram-positive; EO, essential oil.

were identified with main compounds represented by carvacrol (67.93%), *p*-cymene (5.40%), and γ-terpinen (4.25%). In the FS, 16 compounds amounting to 99.62% of the total components were identified, which included carvacrol (83.37%), γ-terpinen (4.13%), and *p*-cymene (3.02%).

The oil contained 66.34%, 86.89%, and 72.49% oxygenated monoterpenes, 22.06%, 10.45%, and 15.02% monoterpene hydrocarbons, 4.26%, 1.72%, and 5.12% sesquiterpene hydrocarbons, and 1.57%, 0.4%, and 3.03% oxygenated sesquiterpenes in early vegetative, late vegetative, and FSs, respectively.

As far as we know, the content of carvacrol in Algerian *O. vulgare* subsp. *glandulosum* EO varied between 18% and 63% according to the geographic origin and time of distillation.[18,19] In Tunisia, Mechergui *et al*.[20] showed that carvacrol content at the FS was 1.7–15.1% according to the population. This may be attributable that the amounts of the EO constituents differ by geographic region, collect time, altitude, and climate. Based on the EO composition and on the classification of *Origanum* taxa previously reported by Kokkini,[21] our *O. vulgare* subsp. *glandulosum* belongs to the carvacrol group. Our results are similar only to that reported on the EO of *O. vulgare* subsp. *glandulosum* grown in cultivation areas in Italy, which showed carvacrol-rich oils (79–64%), γ-terpinene (6–7%), *p*-cymene (5%), and thymol (0.1–5%) as the other main constituents.[22]

The highest content of carvacrol as a major component (83.37%) was observed in the FS. The lowest content of carvacrol was observed in the vegetative stage, but during flower development, the amount is increased (67.93%). During the FS, the highest and the lowest amounts of phenolic compounds (carvacrol + thymol) and their precursors (*p*-cymene + γ-terpinene) were observed, respectively. At vegetative set stages, the amount of phenolic portion was decreased, but the amount of their precursors increased (Table 1). Our result was similar to those reported by Ebrahimi *et al*.[23]

Carvacrol is responsible for the biological activities of oregano. Many diverse activities of carvacrol such as antitumor, antimutagenic, antigenotoxic, analgesic, antispasmodic, antiinflammatory, angiogenic, antiparasitic, antiplatelet, AChe inhibitory, antielastase, insecticidal, antihepatotoxic, and hepatoprotective activities and uses such as feed additive, in honeybee breeding and in gastrointestinal ailments have been shown.

Antimicrobial activity of essentials oils

The antibacterial activity of *O. vulgare* subsp. *glandulosum* EOs against microorganisms, which are considered in this study was assessed by evaluating the presence of inhibition zone and MIC values. Results showed that the EOs of *O. vulgare* subsp. *glandulosum* have great potential of antibacterial activity against all of the five bacteria tested (Table 2). The highest activity was observed against *E. coli* with the strongest inhibition zones (18, 22, and 23 mm) recorded for late, early, and flowering stages, and *S. typhimurium* with larger zones of inhibition (15, 22, and 22.5 mm). However, the antibacterial activity of the EOs was also good except for the ampicillin-resistant *P. aeruginosa* (<15 mm zones of inhibition). Our results

Table 3. Minimum Inhibitory and Bactericidal Concentrations of Essential Oils Against Selected Bacterial Strains

Bacterial strain	EOs (MIC, MBC, μg/mL)							
	CON+		EVS		LVS		FS	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli ATCC 8739 G(–)	15.625	31.25	250	350	250	350	250	400
S. typhimurium ATCC 14028 G(–)	7.8125	15.625	250	300	250	300	250	600
Ampicillin-resistant *P. aeruginosa* (NCTC 10418) G(–)	0.488	0.488	125	400	125	400	125	400
S. aureus ATCC 6538 G(+)	1.95	3.90	250	350	250	350	300	500
B. subtilis G(+)	15.625	15.625	125	250	125	125	125	250

MIC was defined as the lowest concentration of the compounds produced >95% growth reduction compared with the growth in the control well.

Gm, gentamicin; AM, ampicillin; MIC, minimum inhibitory concentration (μg/mL); MBC, minimum bactericidal concentration (μg/mL).

were similar to those previously reported by Hussain *et al*.[24] Against *E. coli* ATCC 8739, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 6538, and *B. subtilis* oils exhibited a high antibacterial activity. However, these bacteria were more susceptible to EOs. The three phenological stages exhibited high amounts of carvacrol (61.03%, 67.93%, and 83.37%).

Screening of pure standard carvacrol on the same microorganisms, exhibited strong antibacterial activity with inhibition zones ranged 11–40 mm and MIC value of 0.22–14.4 mg/mL. No considerable differences between the antibacterial activity of carvacrol and various oils of plants in phenological stages were observed, which could be attributed for high amount of this component in all of the oils.[23] Compared with the positive antibacterial standards, the EOs of *O. vulgare* subsp. *glandulosum* and their main components have a stronger antibacterial activity.

The bacteriostatic and bactericidal effectiveness of the oils at three phonological stages estimated by MIC and MBC, respectively, are shown in Table 3. The findings of this study were in accordance with the antibacterial properties of several *Origanum* species reported previously.[25–27] EOs of *Origanum* species generally contain monoterpenes, carvacrol, thymol, terpinene-4-ol, and linalool. The biological activity of these oils is often attributed to the occurrence of such bioactive compounds.[6,28]

All bacteria were found to be sensitive to all investigated oils. All oils exhibited a high antibacterial activity against *E. coli* and *S. typhimurium* (MIC = 250 μg/mL). Against *P. aeruginosa* and *B. subtilis*, oils at three phenological stages showed a high MIC (MIC = 125 μg/mL). For *S. aureus*, the highest MIC value was observed for the EO at EVS and LVS (Table 3).

Oils during the EVS and LVS showed a very high bactericidal effect. However, oils showed a high bactericidal activity (MBC = 300–400 μg/mL) against the Gram-negative bacteria *E. coli*, *S. typhimurium*, and *P. aeruginosa*. The lowest MBC values have been detected against the two Gram-positive bacteria *S. aureus* and *B. subtilis*.

EOs rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity.[29] Carvacrol, which is the main component of *O. vulgare* subsp. *glandulosum* EOs, has been considered as a biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death.[30,31] The effect of carvacrol on *Staphylococus* was investigated by Knowles *et al*.[32] However, it was also considered that minor components, as well as a possible interaction between the substances could also affect the antimicrobial activities. In fact, other constituents, such as γ-terpinene, have been considered to display relatively good activity due to their possible synergistic or antagonistic effects,[33,34] which is in agreement with our results showing that low amounts of γ-terpinene during the flowering phase may justify the low antimicrobial activity during this period.

CONCLUSIONS

Our study on Tunisian *O. vulgare* subsp. *glandulosum* demonstrates a high variation in the composition of oils and their biological activity potentials at three phonological stages. Thus, the selection of suitable *O. vulgare* subsp. *glandulosum* for chemical traits and/or biological properties can be achieved relatively easy. Thus, the relationship among differences of oils and their biological activities during the phonological stages should be assessed. Tunisian *O. vulgare* subsp. *glandulosum* were chemically distinct and showed a high yield of oils rich in carvacrol, *p*-cymene, and γ-terpinene with a high antibacterial activity. They appear to be interested for selection to promote their culture. The data presented confirm the antibacterial potential of *O. vulgare* subsp. *glandulosum* EO. The EOs tested represent an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent the growth of bacteria and extend the shelf life of the processed food. However, further research is needed to evaluate the effectiveness of *O. vulgare* subsp. *glandulosum* EOs in food ecosystems to establish their utility as natural antimicrobial agents in food preservation and safety.

ACKNOWLEDGMENTS

This research was supported by a grant from the Ministry of Scientific Research and Technology and the National Institute of Applied Science and Technology (Research grant 99/UR/09-10). Authors' Contributions: Afef Béjaoui, Abdennacer Boulila, Hédia Chaabane, and Maroua Jemli have carried out the experimental part such as selection of EOs, injections, and identification of EOs, inoculum preparation and antimicrobial evaluation. Mohamed Boussaid supervised the work, evaluated the results, and corrected the manuscript for publication. All authors read and approved the final manuscript.

AUTHOR DISCLOSURE STATEMENT

The author(s) declare that they have no competing interests.

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