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In vitro antimicrobial activity of essential oil from endemic *Origanum minutiflorum* on ciprofloxacin-resistant *Campylobacter* spp.

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Abstract

This study evaluated the antimicrobial activities of an essential oil of *Origanum minutiflorum* (O. Schwarz and P.H. Davis) against ciprofloxacin-resistant *Campylobacter* spp., by broth microdilution and agar well-diffusion methods. Moreover, *O. minutiflorum* oil was analyzed by gas chromatography/mass spectrometry (GC/MS). Twenty-nine components were identified, representing 98.7 of the oil. The oil yield from the plants was 4.0–4.4% v/w. The major components of *O. minutiflorum* oil were carvacrol (73.9%) and *p*-cymene (7.20%). The oil has lower contents of carvacrol methyl ether (0.05%), heptadecanol (0.06%) and carvacryl acetate (0.06%). Twenty-one *Campylobacter* spp. (12 *C. jejuni*, 5 *C. lari* and 4 *C. coli*) strains using in this study were selected among 300 isolates according to their resistance to ciprofloxacin. The minimum inhibitory concentration (MIC) values for bacterial strains, which were sensitive to the essential oil of *O. minutiflorum*, were in the range of 7.8–800 μg/ml. The essential oil obtained showed strong antimicrobial activity against all of the tested ciprofloxacin-resistance *Campylobacter* spp. These results suggest that the essential of *O. minutiflorum* may be used as a natural preservative in food against food-born disease, such as Campylobacteriosis.

Keywords: Antimicrobial activity; *Origanum minutiflorum*; *Campylobacter* spp.; GC/MS analysis

1. Introduction

Turkey is regarded as an important gene-centre for the family *Lamiaceae*. The leafy parts of plants, such as oregano, thyme and savory, belonging to the *Lamiaceae* family, have been added meat, chicken and food products for many years (Baydar, Sagdic, Ozkan, & Karadogan, 2004). Members of the genus *Origanum* (*Lamiaceae* family) are among the most important aromatic plants worldwide. Twenty-four species and 27 taxa are found in the flora of Turkey and the East Aegean Islands, 16 of them being endemic (Aligiannis, Kalpoutzakis, Mitaku, & Chinou, 2001; Davis, Mill, & Tan, 1984; Davis, Mill, & Tan, 1988). Many *Origanum* plants are characterized by a wide range of volatile secondary metabolites and by the existence of chemical differences with respect to both essential oil content and composition. *Origanum* plants are widely used in the flavouring of food products and alcoholic beverages, as well as in perfumery, because of their spicy fragrance. Moreover, in particular, owing to the antioxidant and antimicrobial activities of their essential oils, *Origanum* species have recently been of great interest, in both academia and the food industry as potential natural additives, to replace synthetic products (Tepe, Daferera, Sokmen, Polissiou, & Sokmen, 2004). Therefore there is an increasing demand for accurate knowledge of the minimum inhibitory concentration (MIC) of essential oils to enable a balance between the sensory acceptability and antimicrobial efficacy (Lambert, Skandamis, Coote, & Nychas, 2001).

Campylobacter spp., especially *Campylobacter jejuni* and *C. coli*, have emerged worldwide as leading causes of acute bacterial gastroenteritis (Threfall, Ward, Frost, & Willshaw, 2000; WHO/FAO, 2001). Several studies have identified chicken as the main source of this infection. *Campylobacter* spp. are part of normal enteric flora in

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animals (chicken, pigs and cattle) and can be transmitted to humans through contaminated foods (Atanassova & Ring, 1999; Dominguez, Gomez, & Zumalacarregui, 2002). Most *Campylobacter* enteric infections are self-limited and do not require antimicrobial drug treatment. However, severe or long-lasting infections do occur and may justify antimicrobial drug therapy. In these cases, erythromycin or fluoroquinolones (e.g. ciprofloxacin) are often the drug of choice (Engberg, Aarestrup, Taylor, Gerner-Smidt, & Nachamkin, 2001). But, antimicrobial resistance, among *Campylobacter* spp., to drugs used in the treatment of human infection is increasing. It is not surprising, therefore, that several countries have reported a rise in ciprofloxacin-resistant *C. jejuni* in human infections (Gaudreau & Gilbert, 2003). Similarly, there has been an increase in the prevalence of ciprofloxacin-resistant *C. jejuni* in human infections, emphasizing the potential to acquire gastroenteritis due to ciprofloxacin-resistant *Campylobacter* from consumption of chicken. (Endtz et al., 1991). Furthermore, some studies have demonstrated an association between ciprofloxacin-resistant *Campylobacter* infections and a longer duration of illness (Engberg, Neimann, Moller Nielsen, Aarestrup, & Fussing, 2004; Nelson et al., 2004). This situation has forced scientists to search for new antimicrobial substances from various sources, such as medical plants (Şahin et al., 2003).

In the literature, there are several studies on antimicrobial activity and the essential oil composition of *Origanum* species, whereas the antimicrobial activity of the essential oil of *Origanum minutiflorum,* against ciprofloxacin-resistant *Campylobacter* spp., has never before been studied. Especially, wild oregano (*O. minutiflorum*) is endemic in Turkey, and so is of special importance for the study. The aims of this study were (i) to investigate the antimicrobial activity of the essential oil of *O. minutiflorum* by broth microdilution and agar well-diffusion methods against ciprofloxacin-resistant *Campylobacter* spp. and (ii) to determine the chemical composition of its hydro-distilled essential oil by GC/MS.

2. Materials and methods

2.1. Plant materials

O. minutiflorum plants were collected during the flowering stage in September, 2004, on Sögüt mountain (elevation 1684 m), Sütçüler-Isparta, where it is endemic. The identification of plant materials was confirmed by a plant taxonomist, Prof. Dr. Hayri Duman, in the Department of Biology, Gazi University, Ankara, Turkey.

2.2. Isolation of essential oil (EO)

A dried sample from the aerial parts (leaves, flowers and steams) of the plant was subjected to water distillation for 3 h in a Clevenger-type apparatus (yield 4.0–4.4% v/w). The EO was stored in the dark at 4 °C prior to further analysis.

2.3. Test microorganisms, isolation and preparation of inocula

Twenty-one *Campylobacter* spp. (12 *C. jejuni*, 5 *C. lari* and 4 *C. coli*) strains were selected among 300 isolates according to their resistance to ciprofloxacin. These strains were isolated from different parts of each of the carcasses: body or cavity. Twenty-five grams from each sample, were placed in 225 ml of pre-enrichment broth (Lab M, Lab 135) in sterile plastic bags for 4 h at 37 °C and 20 h at 42 °C. Following pre-enrichment, 100 μl of the pre-enrichment broth were cultured on *Campylobacter* blood-free agar, containing CCD-agar (charcoal cefoperazone deoxyholate agar) (Lab M, Lab 112 containing vancomycin, polimyxin and trimethoprim). CCD-agar plates were incubated at 42 °C for 48 h in a microaerobic atmosphere, using gas-generating sachets (Oxoid BR 038). *Campylobacter* species were identified by their morphological and Gram stain characteristics (Adesiyum, 1993; Fraser, Chandan, Yamazaki, Brooks, & Garcia, 1992). Isolates were identified as *C. coli*, *C. jejuni* or *C. lari*, using biotyping (api-CAMPY, bio-Merieux).

2.4. Determination of minimum inhibitory concentration (MIC)

Microdilution broth susceptibility assay was used (Koneman, Allen, Janda, Scherckenberger, & Winn, 1997). A stock solution of essential oil was prepared in 10% dimethylsulfoxide (DMSO) and then serial dilutions of essential oil were made in a concentration range from 7.8 to 800 μg/ml. The 96-well plates were prepared by dispensing, into each well, 95 μl of Mueller-Hinton broth (MHB), 100 μl of EO and 5 μl of the inoculants. The inoculums of microorganisms were prepared using 24 h cultures and suspensions were adjusted to 4 McFarland standard turbidity. The final volume in each well was 200 μl. A positive control (containing inoculum but no EO) and negative control (containing EO but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 42 °C for 24 h under microaerophilic conditions (BBL GasPak System). Three replicates of each microassay were carried out and the experiment was carried out twice. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. The experiment was performed in triplicate.

2.5. Inhibitory effect by the agar well-diffusion method

The determination of the inhibitory effect of EO on test bacteria was carried out by the agar diffusion method (Kalemba & Kunicka, 2003). *Campylobacter* cultures were grown at 42 °C for 48 h in MHB. The culture suspensions

were adjusted by comparing against 4 McFarland. Petri dishes with 10 ml of MHA were prepared, previously inoculated with 50 μl of the culture suspension. The wells (∅ 7.0 mm) were made and the EO, diluted in ethanol to the test concentration (10% and 15%), was added to wells (25 μl) and same volume (25 μl) of ethanol was used as a control. The inoculated plates were incubated at 42 °C for 48 h under anaerobic conditions (BBL GasPak System). After incubation, the diameter of the inhibition zone was measured with calipers. The measurements were done basically from the edge of the zone to the edge of the well.

2.6. Gas chromatography/mass spectrometry (GC/MS) analysis conditions

The chemical composition of the essential oil was analyzed using the GC–MS technique. The mass spectrometer was Agilent 6890N GC/5973MSD-SCAN in the electron impact (EI) ionization mode (70 eV) and with an HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxene, 30 mm × 0.25 mm, coating thickness 0.25 μm) Injector and detector temperatures were set at 220 °C. The oven temperature was held at 50 °C for 30 min, then programmed to 240 °C at rate of 3 °C/min. Helium (99.99%) was the carrier gas at a flow rate of 1 ml/min. Diluted samples (1/100 in hexane, v/v) of 1.0 μl were injected manually (Aligiannis et al., 2001). The identifications of the components were based on the comparison of their mass spectra with those of Wiley 7N (contains 392086 compounds spectra), Nist 2002 (contains 174948 compounds spectra) and Flavour (contains 419 compounds) spectra Libraries, as well as by comparison of their retention times.

2.7. Statistical analysis

All experiments were done in triplicate, and mean values are presented. Statistical analysis was performed on the data by SPSS 11.0 Bivariate Correlation Analysis (SPSS Inc., Chicago, Ill.) with statistical significance determined at $P < 0.01$. The Pearson rank order correlation test was used for comparisons both of broth microdilution and agar well-diffusion methods, of the antimicrobial activity of the essential oil.

3. Results and discussion

Antimicrobial resistance, in both medicine and agriculture, is recognized by the World Health Organization (WHO), along with various other national authorities, as a major emerging problem of public health importance. Since *Campylobacteriosis* is transmitted primarily through food, the presence of antimicrobial-resistant *Campylobacter* in raw meat products has important public health implications especially in developing countries, where there is widespread and uncontrolled use of antibiotics (Hart & Kariuki, 1998). Antimicrobial resistance, particularly against the fluoroquinolones (e.g. ciprofloxacin) antibiotics, has now emerged globally with thermophilic *Campylobacters*. Recently, the acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to the available antibiotics have led authors to investigate the antimicrobial activity of medicinal plants. Our interest is focussed on the effectiveness of *O. minutiflorum* essential oil against ciprofloxacin resistance *Campylobacter* spp.

The percentage composition of the oil of *O. minutiflorum* is presented in Table 1. Twenty-nine components were identified, accounting for 98.7% of the oil. The analysis showed that carvacrol (73.9%) was the main component in the oil of *O. minutiflorum*. Other major components were identified as *p*-cymene (7.20%), γ-terpinene (3.99%) and borneol (2.41%). The oil has lower contents of carvacrol methyl ether (0.05%), heptadecanol (0.06%) and carvacryl acetate (0.06%). *Origanum* spp. were previously tested on various pathogenic bacteria. Studies showed that *Origanum scabrun*, which contains 75% of carvacrol, had a very high antibacterial effect against *S. aureus* and *Escherichia coli* (Aligiannis et al., 2001). A recent study has shown that the essential oil of *Origanum vulgare* is characterized principally by phenol constituents, thymol and carvacrol (24.7 and 14.0% of the total oil, respectively) and by their two precursors monoterpene hydrocarbons, γ-terpinene

Table 1
Chemical composition of *O. minutiflorum* essential oil

Compounds	RT min	Composition (%)
1. α-Thujene	8.297	0.90
2. α-Pinene	8.528	0.77
3. Camphene	9.083	0.65
4. β-Pinene	10.209	0.18
5. 1-Octen-3-ol	10.406	0.12
6. Myrcene	10.891	0.97
7. α-Phellandrene	11.395	0.17
8. δ-3-Carene	11.649	1.05
9. α-Terpinene	11.937	0.83
10. *p*-Cymene	12.318	7.20
11. β-Phellandrene	12.469	0.49
12. 1.8 Cineole	12.572	0.37
13. β-Ocimene	12.954	0.11
14. γ-Terpinene	13.853	3.99
15. α-Terpinolene	15.171	0.13
16. Borneol	18.717	2.41
17. Terpineol	19.357	0.63
18. Carvacrol methyl ether	22.376	0.05
19. Carvone	23.313	0.13
20. Thymol	24.721	0.28
21. Carvacrol	25.582	73.93
22. Carvacryl acetate	28.087	0.06
23. β-Caryophyllene	29.971	1.93
24. Aromadendrene	30.738	0.24
25. α-Humulene	31.336	0.10
26. Ledene	33.036	0.18
27. Spathulenol	36.247	0.28
28. Caryophyllene-oxide	36.450	0.51
29. Heptadecanol	53.434	0.06
Total		98.72

and *p*-cymene (11.7 and 14.6% of the total oil, respectively) (Nostro, Blanco, Cannatelli, Flamini, & Morelli, 2004). In addition, no antimicrobial activity has been reported for *p*-cymene or γ-terpinene (Aligiannis et al., 2001; Sivropoulou et al., 1996). The chemical composition of essential oils depends on climatic, seasonal, and geographic conditions (Baydar et al., 2004). Recently, there has been considerable interest in essential oils from aromatic plants with antimicrobial activities for controlling pathogens and/or toxin-producing microorganisms in foods (Alzoreky & Nakahara, 2003; Valero & Salmeron, 2003). In addition, their antimicrobial activity depends on the type, composition and concentration of essential oils. Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity (Aligiannis et al., 2001; Baydar et al., 2004). The antimicrobial activities of the essential oil of *O. minutiflorum* against ciprofloxacin-resistant *Campylobacter* spp. examined in the present study and their potency were assessed by the inhibition zone diameter, and MIC values. These results are given in Table 2. The inhibition zones and MIC values for *Campylobacter* spp. strains which were sensitive to the essential oil of *O. minutiflorum*, were in the range 10–28 mm (1/10 diluted with ethanol) and 7.8–800 μg/ml, respectively. The largest inhibition zones produced by the essential oil were observed with strains 102a and $6t_1$ of *C. lari* (>28 mm). However, the highest inhibitory activity was against *C. lari* 102a and $6t_1$ strains which showed the lowest MIC (7.8 μg/ml) and largest growth inhibition halos. The inhibition zones of the essential oil on *Campylobacter* spp. strains showed a significant correlation with MIC values ($P < 0.01$). Several studies have reported the essential oils effects on *Campylobacter* strains during recent years. Freidman, Henika, and Mandrell (2002) studied plant essential oil (27 oils and 12 compounds) bactericidal activities against *C. jejuni*, *E. coli*, *Listeria monocytogenes* and *Salmonella enterica*. They found that the essential oils were most active against *C. jejuni* (Freidman et al., 2002). On the other hand, *C. jejuni* was shown to be resistant to 21 essential oils by other researchers (Smith-Palmer, Steward, & Fyfe, 1998). On the other hand, an essential oil of *O. minutiflorum* showed effective antimicrobial activity against all ciprofloxacin-resistance *Campylobacter* spp. in this study.

Table 2
MIC values of *Origanum minutiflorum* essential oil against *Campylobacter* spp. tested in micro-well dilution assay

Campylobacter spp.	MIC (μg/ml)	Inhibition zone diameter (mm)	
		1/10	1/15
C. jejuni			
118d	12.5	22 ± 1	17 ± 1
121a	31.2	27 ± 0	27 ± 0
116e	62.5	25 ± 0	21 ± 0
113k	700	13 ± 1	10 ± 1
117j	125	21 ± 0	18 ± 1
$6t_4$	31.2	28 ± 0	28 ± 0
17a	62.5	24 ± 0	21 ± 0
G6	31.2	27 ± 0	25 ± 0
2c	125	20 ± 0	17 ± 0
14a	125	22 ± 0	18 ± 0
7d	700	12 ± 1	9 ± 0
9a	700	13 ± 1	11 ± 0
C. lari			
$6t_1$	7.8	>28	>28
$6t_5$	800	11 ± 0.4	8 ± 0
$13t_1$	500	17 ± 0.3	15 ± 0
102a	7.8	>28	>28
114d	31.2	27 ± 0	25 ± 0
C. coli			
108c	125	22 ± 1	18 ± 1
120f	31.2	28 ± 0	26 ± 0
$13t_5$	250	18 ± 0	16 ± 0
14b	800	10 ± 0	9 ± 0

Values represent averages ± standard deviations for triplicate experiments.

The essential oil of *O. minutiflorum* can be used as a natural preservative in food against food-born disease and food spoilage of *Campylobacter* spp. Essential oils, as antimicrobial agents present two main characteristics: the first is their natural origin which means more safety for consumers and the second is that they are considered to be low risk for resistance development by pathogenic microorganisms. Our study may be considered as the first report on the antimicrobial activity of an essential oil against ciprofloxacin-resistant *Campylobacter*. We hope that our results will provide a starting point for investigations designed to exploit new natural antimicrobials effective against *Campylobacter*.

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