JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 1 (2014) 120-124

Phytochemical and Antibacterial Properties of *Origanum vulgare* ssp. *gracile* Growing Wild in Kurdistan Province of Iran

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Article type
Original article

Keywords
Origanum
Phytochemicals
Anti-Bacterial Agents

Received: 3 Oct 2014
Revised: 1 Nov 2014
Accepted: 26 Nov 2014

Abstract

Background: In current study, chemical composition and antibacterial activity of leaves and flowers essential oils of *Origanum vulgare* ssp. *gracile* against four food-borne pathogens including *Escherichia coli*, *Listeria monocytogenes*, *Salmonella thyphimurium* and *Staphylococcus aureus* were examined.

Methods: The different organs (leaves and flowers) of *O. vulgare* ssp. *gracile* were harvested at flowering stage from wild grown plants in Kurdistan province, Iran. Phytochemical properties of the essential oils were identified by GC and GC/MS analysis. Also, antibacterial effects of *O. vulgare* ssp. *gracile* against each bacterial strain were detected by calculating the minimum inhibitory concentration and minimum bactericidal concentration using microdilution method. Data was analyzed by chi square and fisher exact tests using SPSS 16.0.

Results: Main components of leaf and flower oils were carvacrol (46.5% and 60.6%), γ-terpinene (13.91% and 16.64%) and ρ-cymene (13.54% and 7.21%). *E. coli* and *S. aureus* had similar sensitivity to essential oils, but *S. typhimurium* showed more sensitivity. Output of composition analysis showed that the *O. vulgare* ssp. *gracile* flowers essential oil has more effect than those from leaves.

Conclusion: This work showed substantial inhibitory effect of *O. vulgare* ssp. *gracile* essential oils on food-borne pathogens. The efficiency of *O. vulgare* ssp. *gracile* flowers oils in reduction of the bacterial activity was higher than the leaves essential oils. According to the results of this research, this essential oil can be utilized as a natural preservative in food products.

Introduction

Recently, many studies have focused on determination of various properties of new natural antimicrobials within plant, animal or microbial sources. This is because of the increase in resistant bacteria to the current antimicrobial agents and side effects of chemical and synthetic antimicrobials (Hashemi et al., 2013; Hussain et al., 2008). Secondary metabolites of plants such as flavonoids, alkaloids and terpenoids are known for their antimicrobial properties (Cowan, 1999; Hashemi et al., 2013). Therefore, essential oils obtained from various parts of plants and their components are as a choice of interest due to their favorable properties and potential functional use (Entezari et al., 2009). Many researchers have reported their strong and functional effects such as *in vitro* antifungal, antimicrobial, and antioxidant effects (Avijgan et al., 2006; Chalchat et al., 2007; Hamzeh, 2012; Hashemi et al., 2013; Proestos et al., 2005; Singh et al., 2011).

The genus *Origanum* (oregano) is one of over 200 genera in the Lamiaceae family and includes annual and perennial herbs growing on stony slopes at a wide range of altitudes

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(Aligiannis et al., 2001; Craker, 1989). It comprises about 38 species, 6 subspecies and 17 hybrids, most of them are indigenous to the Mediterranean region (Ietswaart, 1980; Vokou et al., 1993).

Most *Origanum* species naturally grow in Eurasia and Mediterranean zone. *Origanum vulgare* L. is widely distributed in the world including the Mediterranean, Irano-Turanian and Euro-Siberian regions. *Origanum vulgare* ssp. *gracile* is a perennial herb with white flower that is native to Afghanistan, Iran, East of Turkey, North of Iraq, Northwest of Pakistan, South and Center of Russia. In Iran, *O. vulgare* contains three subspecies including ssp. *viride*, ssp. *vulgare* and ssp. *gracile*. The distribution area of this subspecies in Iran is in north and west provinces such as Gilan, Mazandaran, Azerbaijan and Kurdistan that grows wildly in these regions (Ietswaart, 1980).

The *Origanum* species, which are rich in essential oils, have been used as spices and in folk medicine of many countries as diuretic, stomachic, antineuralgic, antitussive, expectorant, sedative, stimulant, carminative and antirheumatic (Afsharypour et al., 1997; Dundar et al., 2008; Vokou et al., 1993). Like any other aromatic crops, the biosynthesis of secondary metabolites of *Origanum* species are mostly variable and affected strongly by interaction between the genotype and environment, climatic, seasonal and geographic conditions, harvest time, method of distillation and storage condition (Figueiredo et al., 2008; Verma et al., 2010). Plant maturity and presence of chemotypic differences at the time of the essential oil preparation can affect the oil composition as well (Tounsi et al., 2011). Nevertheless, many of the studies confirmed their antibacterial, antifungal and antioxidant activities (Bakkali et al., 2008; Kulisic et al., 2004).

Recent studies have shown that the essential oil of *O. vulgare* exhibited optimum inhibitory effects on food-borne pathogens such as *Salmonella* (Friedman et al., 2002; Nevas et al., 2004; Penalver et al., 2005). Oregano owes its activity to the essential oil in glandular hairs and the major components are phenolic monoterpenoides mainly carvacrol and occasionally thymol (D'Antuono et al., 2000). So that accomplished researches in this regard have proven that antibacterial activity of the essential oils of *Origanum* is mainly based on the destruction of the bacterial cell membrane (Burt, 2004).

O. vulgare ssp. *gracile* is a native plant in Iran that the chemical composition and further properties of its essential oil obtained from various parts of this plant has not been studied, yet. Hence, the general objectives of this study were 1) to determine the chemical composition of hydro-distilled essential oils of *O. vulgare* ssp. *gracile* and 2) to compare the antibacterial activities and potential bactericidal properties of aerial parts of this plant (leaves and flowers).

Materials and methods

Plant material

The different organs (leaves and flowers) of *O. vulgare* ssp. *gracile* were harvested at flowering stage in mid-July 2011 from wild grown plants in the Saral area, Zardavan village district of Kurdistan province, North-West of Sanandaj city, Iran. A voucher specimen (no. 9428) was deposited in the Herbarium of the Agriculture and Natural Resources Center of Kurdistan province.

Extraction of essential Oil

Dried leaves and flowers of *O. vulgare* ssp. *gracile* were separated in room temperature from the other parts of plant and subjected to the hydro distillation for 3 h using a clevenger-type apparatus, according to the method recommended by the European Pharmacopeia (Ahmad et al., 1999; Akgül and Chialva, 1989). The obtained essential oils was dried over anhydrous sodium sulphate and stored in sterilized dark glass at 4 ˚C for further experiments.

Essential oil analysis

Essential oil constituents were determined by GC and GC/MS analysis. GC analysis was carried out on a Shimadzu 9A gas chromatograph equipped with a Ph-5 column (30 m×0.1 mm, film thickness 0.25 µm). Oven temperature was held at 60 ºC for 5 min and then programmed to 250 ºC at a rate of 3 ºC/min and kept constant at 250 ºC for 10 min. Injector and detector temperature was adjusted to 260 ºC and helium was used as carrier gas with a linear velocity of 32 cm/s. Data were calculated by electronic integration without using response correction factor GC/MS analysis was also carried out on a varian 3400 GC/MS system equipped with a DB-5 fused silica column (30 m×0.25 mm, film thickness 0.25 µm). Oven temperature program was 50–250 °C at a rate of 4 °C/min, transfer line temperature 260 °C, carrier gas was helium with a linear velocity of 31.5 cm/s, split ratio 1/60, ionization energy 70 eV. The components of the oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds, confirmed by comparison of their retention indices (RI) with those of authentic compounds or with data published in literatures (Davies, 1990).

Bacterial strains

The essential oils antimicrobial effects were studied on two Gram-negative (*Salmonella thyphimurium* ATCC 13311 and *Escherichia coli* ATCC 43894) and two Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC 19118), individually. Lyophilized cultures of the organisms were obtained from the culture

collection of the Department of Food Hygiene, Faculty of Veterinary Medicine, University of Urmia, Urmia, Iran.

Micro-well dilution assay

The MIC and MBC values of the essential oils were studied for the bacterial strains in micro-plates. Bacterial strains were prepared from 18 h nutrient broth cultures and suspensions were conformed to 0.5 McFarland standard turbidity. Essential oils were dissolved in 10% dimethyl sulfoxide (DMSO; Sigma–Aldrich) and the obtained solutions firstly were diluted to the highest concentration (10000 ppm) as a stock solution and then serial two-fold dilutions were made in a concentration range from 625 to 10000 ppm in nutrient broth. MIC values of essential oils against bacterial strains were determined based on a micro-well dilution method. The 96-well plates were prepared by distributing 160 µl nutrient broth and 20 µl inoculums (10^6 CFU/ml) into each well.

A 20 µl aliquot from the stock solutions of essential oils prepared initially at the concentration of 10000 ppm, were added into the first wells. Then, 20 µl of their serial dilutions were transferred into consecutive wells. The last well contained 180 µl nutrient broth without any chemical compounds and 20 µl inoculum on each strip and used as the negative control. The final volume in each well was 200 µl (Weerakkody et al., 2010). Thus, the final concentrations of essential oils were between 62.5 to 1000 ppm and bacterial suspensions were approximately 10^5 CFU/ml. All experiments were replicated at least 3 times. The plates were covered with a sterile plate cap. Contents of plate were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures (37 °C for 24 h). Microbial growth was assessed by optical absorbance measurement at 600 nm using the EL×800 universal micro-plate reader (Biotek Instrument Inc, Highland Park, Vermont, USA) and verified by plating 10 µl samples from clear wells on nutrient agar medium. The MIC and MBC were defined as the lowest concentration of the essential oils to inhibit the growth of microorganisms and show bactericidal effects on microorganisms, respectively (Bagiu et al., 2012; Hussain et al., 2008).

Statistical analysis

Data was analyzed by SPSS software (16.0) at 5% significance level. In order to analyze the data, the chi square test, fisher exact test and logistic regression were used. Firstly, statistical analysis was performed to determine significant differences ($p \leq 0.05$). Then, by uni-variant logistic regression and odds ratios, the roles of each factor were determined.

Results

Leaves and flowers of the *O. vulgare* ssp. *gracile* are different in their essential oils content with 1.44% and 2.44%, respectively. The results of GC and GC-MS analysis of the essential oils are presented in Table 1. The main components determined in both leaves and flower essential oils were carvacrol (46.5% and 60.6%), γ-terpinene (13.91% and 16.64%) and ρ-cymene (13.54% and 7.21%), respectively.

Antibacterial activity of leaves and flowers essential oils from oregano plant against four different bacteria that are known to cause food-borne diseases are presented in Table 2. Significant difference was observed between antibacterial properties of the essential oils obtained from leaves and flowers of *O. vulgare* ssp. *gracile* plant. The efficacy of flowers oil in bacterial activity reduction was higher than that of the leaves essential oil. Therefore, the odd of inhibition of food-borne pathogens was 3.45 times higher in wells containing flowers essential oil than leaves essential oil ($p \leq 0.05$) and 11.6% of changes in anti-bacterial properties were justified by selection of appropriate part of plant.

Discussion

According to the results of GC/MS analysis (Table 1), carvacrol and thymol were determined as major components of tested essential oil and these components believed to be the main sources of antioxidative, antimicrobial and antifungal effect of oregano oil (Bakkali et al., 2008; D'Antuono et al., 2000; Kulisic et al., 2004). As shown in Table 2, the essential oils showed variable antibacterial activities against studied bacteria. Several studies have indicated that the chemical compositions of the essential oils obtained from various parts of plants are different (Arcila-Lozano et al., 2004; Kovacevic et al., 2007; Proestos et al., 2005). A significant increase in the inactivation rate of bacteria was observed with rising concentration of essential oils (flowers and leaves), but no significant correlation was observed between the inhibitory and bactericidal effects of essential oils and various types of bacteria strains (Gram-positive and Gram-negative). *E. coli* and *S. aureus* had similar sensitivity to essential oils, but *S. typhimurium* showed more sensitivity. Nevas et al. (2004) reported greater sensitivity of Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) than Gram-negative bacteria (*S. typhimurium* and *E .coli*) to oregano essential oils. However, Di Pasqua et al. (2005) reported that *L. monocytogenes* and *S. aureus* were more resistant compared to *S. typhimurium* and *E. coli*. The differences observed in the results of several researchers could be related to the presence of various strains of bacteria for micro-dilution test or different chemical compounds of essential oils, due to plant growth in different environmental conditions (Burt, 2004).

Table 1: Essential oil components of various parts of *O. vulgare* ssp. *gracile*

No.	Components	RI	Percentage in the oil	
			Leaf	Flower
1	α-thujene	928	0.72	1.45
2	α-pinene	937	0.47	0.85
3	Octen-3-ol	983	0.5	0.26
4	3-octanone	991	3.5	2.89
5	α-phellanderene	1005	0.24	0.46
6	α-terpinene	1015	1.23	2.39
7	ρ-cymene	1025	13.54	7.21
8	1,8-cineol	1033	2.76	-
9	(Z)-β-ocimene	1044	0.56	0.28
10	γ-terpinene	1058	13.91	16.64
11	Terpinolene	1081	0.14	0.12
12	Cis- ρ -menth-2-en-1-ol	1118	0.47	-
13	Terpinene-4-ol	1161	0.94	0.63
14	α-terpineol	1172	1.07	0.29
15	Thymol methyl ether	1225	0.15	-
16	Carvacrol methyl ether	1236	7.19	2.04
17	Thymol	1291	2.24	1.82
18	Carvacrol	1304	46.5	60.6
19	E-caryophyllene	1408	0.85	0.35
20	Germacrene D	1497	0.24	0.1
21	Germacrene A	1505	0.19	-
22	γ-elemen	1526	2.35	1.21
23	Spathulenol	1555	0.22	-
	Total		99.98	99.59

Table 2: Antibacterial properties of flowers and leaves essential oils from *O. vulgare* ssp. *gracile* against four tested bacteria in this study

Strains	Flower essential oil		Leaf essential oil	
	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)
S. thyphimurium	125	250	500	500
S. aureus	250	500	500	500
L. monocytogenes	250	500	1000	1000
E. coli	250	250	500	500

Due to the existence of different chemical components, antimicrobial effect of essential oils is not directed to a specific mechanism but, mechanisms of essential oils against bacteria include disruption of the cell walls, destroying of cytoplasmic membrane and membrane proteins, loss of cell contents, coagulation in the cytoplasm, and dysfunction of the system activated proton transfer. Among the mentioned mecahnisms, it seems that membrane damage is the most important inhibitory effect of oregano essential oils (Bakkali et al., 2008; Burt, 2004).

In addition, the essential oils of *O. vulgare* ssp. *gracile* can potentially be used in food products to eliminate pathogenic and spoilage bacteria, which limits the potential of continuous contamination of food during processing. Prospects for development of improved approaches against antibiotic resistance bacteria are promising. Essential oils could become very valuable in the control of gastrointestinal infection through reducing the number of resistant bacteria to antibiotics in food products.

Conclusion

Growth inhibitory effect of essential oils from *O. vulgare* ssp. *gracile* on food-borne pathogens was verified in this study. The efficacy of flower oil from *O. vulgare* ssp. *gracile* in reducing of the bacterial activity was higher than the leaves essential oils. According to these results, the essential oils obtained from flower of *O. vulgare* ssp. *gracile* can be utilized as a natural preservative in food products.

Conflicts of interest

The authors had no conflict of interest in this research.

Acknowledgement

We wish to thank the Research Vice Chancellor of Urmia University, Urmia, Iran for financial supports of this study.

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