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Inhibitory Activity and Chemical Characterization of *Daucus carota* subsp. *maximus* Essential Oils

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The essential oils (EOs) of green seeds from *Daucus carota* subsp. *maximus* growing wild in Pantelleria Island (Sicily, Italy) were characterized. EOs were extracted by steam distillation, examined for their inhibitory properties against food-borne *Gram*-positive and *Gram*-negative bacteria and analyzed for the chemical composition by gas chromatography (GC) and mass spectrometry (MS). Undiluted EOs showed a large inhibition spectrum against *Gram*-positive strains and also *vs. Acinetobacter* spp. and *Stenotrophomonas maltophilia*. The minimum inhibition concentration (*MIC*) was in the range 1.25 – 2.50 μl/ml for the most sensitive strains. The chemical analysis indicated that *D. carota* subsp. *maximus* EOs included 34 compounds (five monoterpene hydrocarbons, six oxygenated monoterpenes, 14 sesquiterpene hydrocarbons, four oxygenated sesquiterpenes, camphorene and four other compounds), accounting for 95.48% of the total oil, and that the major chemicals were carotol, β-bisabolene, and isoelemicin.

Keywords: Chemical composition, *Daucus carota*, Essential oils, Inhibitory activities, Foodborne bacteria.

Introduction

The recent awareness of consumers towards the health implications of chemical preservatives in foods has greatly stimulated the addition of natural biopreservatives to ensure their safety. Essential oils (EOs) and extracts of various plants, herbs, spices, and fruits show antimicrobial potential[1][2] useful to inhibit undesired pathogenic and spoilage microorganisms harboured in foods. The composition of EOs includes a complex mixture of several compounds and the presence and concentration of some active constituents determines the inhibition spectrum. These plant extracts are referred to as GRAS (Generally Recognised As Safe)[3]. Thus, their incorporation in foods encounters the growing request of natural alternative to chemical preservatives. The necessity to apply valid substitutes of synthetic chemical compounds is also related to the emergence of human bacterial pathogens which are resistant to classical preservatives.[4]

Indeed, there is an open debate in the scientific community about the extensive use of EOs in foods. In fact, the antimicrobial potential of EOs is not enough to justify their addition during food manipulation. As a matter of fact, a given EO represents a food ingredient and the attention of the food scientists on this topic is high, since some EOs are characterized by cancer-causing effects[5] with adverse consequences for the consumers.

Among the food-borne diseases, one of the major microbial threats is still represented by listeriosis.[6] Although *Listeria monocytogenes* has a very low incidence, this bacterium shows a high case fatality rate exceeding 30%.[7] Furthermore, several industrialized countries registered an increasing incidence of this disease among persons aged 65 years and older.[8] Several EOs from wild plants have been recently characterized for their antilisterial activities in order to select new biopreservatives. Among these plants, members of the *Apiaceae* family are gaining more and more interest.[9][10]

Within *Apiaceae* family, the species *Daucus carota* subsp. *maximus* has not been characterized for the antibacterial properties of its EOs yet. Not even the chemical composition of the EOs of *D. carota* subsp. *maximus* plants has been extensively investigated. *Valente et al.*[11] analyzed the EOs extracted from the

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ripe umbels of plants of this species collected in south Portugal, while *Saad et al.*[12] characterized the EOs from the fruits, leaves and stems of plants grown in experimental fields in Mansoura (Egypt). So far, no information is available on the chemical compounds that characterize the EOs of plants growing in sites located in the central Mediterranean islands.

Based on the above considerations and in order to acquire information on the EOs extracted from the seeds of *D. carota* subsp. *maximus*, the objectives of this study were *i*) to evaluate the inhibitory potential of the EOs extracted from the green seeds of plants growing wild in a southern Mediterranean site against *L. monocytogenes* and other several common food-borne pathogens and *ii*) to determine their chemical composition.

Results and Discussion

Antibacterial Activity of Daucus carota *subsp.* maximus *EOs*

The common approach to study the positive features of EOs in view of their food application is often forwarded to the evaluation of the inhibitory activities against food-borne pathogens.[13] In this study, the inhibitory spectrum of the EOs extracted from the green seeds of *D. carota* subsp. *maximus* was characterized against bacterial species (*Table 1*), mainly *L. monocytogenes* that is responsible for human disease commonly associated with the consumption of contaminated food items[14][15] and other *Gram*-positive and several *Gram*-negative (mostly belonging to the *Enterobacteriaceae* family) species that have been isolated from food sources and reported to be the cause of infection in humans.

The EOs were highly effective against 18 out of the 25 *L. monocytogenes* tested with a diameter of the inhibition area around the paper disc in the range 9 – 12 mm for 16 strains and higher than 13 mm for *L. monocytogenes* DHPS133 and DHPS179. Only six *L. monocytogenes* strains showed a clear growth resulting from being resistant to the EOs. In general, the majority of the *Gram*-positive strains were inhibited by *D. carota* subsp. *maximus* EOs. In particular, all strains of *Staphylococcus* were particularly sensitive to these inhibitory compounds that showed a strong activity. The *Staphylococci* used in the present study belong to the coagulase negative group. However, they can be relevant pathogens: *Staphylococcus epidermidis*, colonizing the skin and mucous membranes of the human body, has become an important cause of nosocomial infections[16]; *Staphylococcus warneri* is responsible for bacteremia in adult and pediatric patients[17]; *Staphylococcus haemolyticus* is an opportunistic pathogen associated to implanted medical devices.[18]

Among the *Gram*-negative bacteria only the *Acinetobacter* strains and *Stenotrophomonas maltophilia* ICE272 were inhibited. *Acinetobacter guillouiae* and *Acinetobacter haemolyticus*, although rare and restricted to catheter-related bloodstream infections, have been reported as agents causing hospital outbreaks,[19] while *St. maltophilia* is an important nosocomial pathogen, especially in debilitated and immunocompromised persons.[20]

In order to retrieve the minimum inhibition concentration (*MIC*), the EOs were serially diluted and further tested against the strains *L. monocytogenes* DHPS179, *S. warneri* ICE20, *Bacillus cereus* ICE170, and *A. haemolyticus* ICE47, which showed the highest sensitivity in terms of the width of the inhibition halos. *D. carota* subsp. *maximus* EOs showed the following *MIC* values: 2.50 μl/ml against *L. monocytogenes* DHPS179 and 1.25 μl/ml against the other three strains. These results showed that *D. carota* subsp. *maximus* EOs tested were active against high concentrations (10^6 CFU/ml) of the sensible strains indicating a defining applicative potential to inhibit microbial food pathogens. In general, the pathogenic microorganisms contaminate foods because they are present in the raw materials or enter the food during manufacturing and also during storage and handling steps.[21] For these reasons, it is evident that the importance of keeping the growth of the above species under control and the use of EOs represents an effective strategy alternative to the use of chemical compounds.

Chemical Analysis

The yield of *D. carota* subsp. *maximus* EOs obtained by steam distillation was 1%. The qualitative and quantitative composition of the EOs are reported in *Table 2*, where the compounds are shown in the order of their elution and classified per phytochemical class. Thirty-seven chemical compounds were identified by GC/MS, accounting for 95.48% of the total oil. The chemicals included monoterpene hydrocarbons (1.78%), oxygenated monoterpenes (7.39%), sesquiterpene hydrocarbons (24.85%), oxygenated sesquiterpenes (48.71%), diterpenes (0.26%), and other compounds (12.49%). The major compounds of the EOs were carotol (44.68%) and β-bisabolene (12.72%) showing a clear dominance of the oxygenated sesquiterpenes and sesquiterpene hydrocarbons over the other phytochemical classes. However, isoelemicin unclassified was

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Table 1. Inhibitory activity of *Daucus carota* subsp. *maximus* EOs

Strains	Inhibition[a]	Source of isolation
Acinetobacter guillouiae ICE24	+	Ice cubes
Acinetobacter haemolyticus ICE47	++	Ice cubes
Bacillus cereus ICE170	+	Ice cubes
Enterobacter ludwigii 4G145	−	Ready to eat salad
Hafnia halvei 4G44	−	Ready to eat salad
Hafnia paralvei 4G53	−	Ready to eat salad
Lactobacillus sakei LMG 2313	+	Unknown
Listeria innocua 4202	±	Unknown
Listeria monocytogenes ATCC 19114	+	Animal tissue
L. monocytogenes DHPS11BO	+	Meat factory
L. monocytogenes DHPS129	+	Human stool
L. monocytogenes DHPS12BO	+	Ripened salami
L. monocytogenes DHPS131	+	Human stool
L. monocytogenes DHPS133	++	Human stool
L. monocytogenes DHPS13BO	±	Gorgonzola cheese
L. monocytogenes DHPS179	++	Salmon
L. monocytogenes DHPS180	+	Ricotta cheese
L. monocytogenes DHPS182	+	Ricotta cheese
L. monocytogenes DHPS184	+	Rice salad
L. monocytogenes DHPS185	+	Beef
L. monocytogenes DHPS186	+	Mozzarella salad
L. monocytogenes DHPS187	−	Roasted chicken
L. monocytogenes DHPS188	−	Green salad
L. monocytogenes DHPS1BO	−	Chopped meat
L. monocytogenes DHPS20BO	+	Gorgonzola cheese
L. monocytogenes DHPS22BO	+	Taleggio cheese
L. monocytogenes DHPS24BO	+	Taleggio cheese
L. monocytogenes DHPS2BO	−	Fresh salami
L. monocytogenes DHPS3BO	−	Fresh salami
L. monocytogenes DHPS4BO	+	Ripened salami
L. monocytogenes DHPS5BO	+	Ripened salami
L. monocytogenes DHPS6BO	−	Ripened salami
L. monocytogenes DHPS7BO	+	Ripened salami
Raoultella ornithinolytica 4G594	−	Ready to eat salad
Serratia grimesii 4G954	−	Ready to eat salad
Staphylococcus epidermidis ICE244	++	Ice cubes
Staphylococcus haemolyticus ICE182	++	Ice cubes
Staphylococcus warneri ICE20	++	Ice cubes
Stenotrophomonas maltophilia ICE272	+	Ice cubes

[a] −, no inhibition; ±, low inhibition (< 9 mm diameter); +, clear inhibition (9 – 12 mm diameter); ++, strong inhibition (> 13 mm diameter). Results indicate the mean value of three independent assays.

detected at a relevant presence (11.51%). Among the oxygenated monoterpenes, geranyl acetate (4.36%) was the main compound followed by 4-carvomenthenol (2.33%). All other compounds were below 2%.

Previous investigations reported a major presence of *trans*-methylisoeugenol, β-bisabolene, and β-asarone in the EOs from the aerial parts of *D. carota* subsp. *maximus* plants grown in Egypt,[12] while geranyl acetate, β-bisabolene, α-asarone, and α-pinene in the EOs from ripe umbels of plants of this species grown in south Portugal.[11] To the best of our knowledge, our study represents the first investigation on the composition of the EOs from the green seeds of *D. carota* subsp. *maximus*. The composition of the EOs found in this study is highly similar to that of the EOs from the seeds of *Daucus carota* L. ssp. *carota* cultivated in Italy, reported to be poor in monoterpens and containing high amount of methyl isoeugenol, β-bisabolene, elemicin, and carotol.[22] The results of this study evidenced a difference in the composition of EOs extracted from different parts of the plants.

Regarding the major compound detected in this study, carotol, it is commonly found at consistent percentages in EOs from different aerial parts (flowers,

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Table 2. Chemical composition of *Daucus carota* subsp. *maximus* EOs

Chemical compound	Retention time [min]	%
Monoterpene hydrocarbons		1.78
α-Pinene	10.707	0.21
Myrcene	12.89	0.19
p-Cymene	13.993	0.69
Limonene	14.127	0.51
Carene	23.447	0.18
Oxygenated monoterpenes		7.39
4-Carvomenthenol	18.926	2.33
Terpineol	19.334	0.40
β-Citronellol	20.522	0.08
Citronellyl formate	21.738	0.07
Isobornyl acetate	22.055	0.15
Geranyl acetate	24.653	4.36
Sesquiterpene hydrocarbons		24.85
δ-Elemene	23.762	0.19
Daucene	24.577	2.34
Caryophyllene	25.615	1.11
Bergamotene	25.989	0.78
Sesquiphellandrene	26.173	0.99
Farnesene	26.487	1.27
Acoradiene	26.987	0.88
Curcumene	27.156	0.73
Selinene	27.287	0.94
β-Bisabolene	27.82	12.72
Muurolene	28.16	0.54
Bisabolene trans alpha	28.586	0.40
Gurjunene	30.712	0.95
Selina-3,7(11)-diene	32.157	1.01
Oxygenated sesquiterpenes		48.71
Caryophyllene oxide	29.638	0.97
Carotol	30.027	44.68
Daucol	30.934	1.43
Isocalamendiol	33.195	1.63
Diterpenes		0.26
Camphorene	37.289	0.26
Others		12.49
2-Cyclohexen-1-one	19.215	0.20
Cyclohexanone	27.886	0.15
Isoelemicin	29.119	11.51
Asarone	30.429	0.63
Total		95.48

Results indicate mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant and common peaks to all samples) × l00.

leaves, stems, and other aerial parts) of *D. carota* L. ssp. *carota*[23] and, of course, at very high concentrations (> 60%) in those extracted from the fruits.[24][25] In fact, carotol is the typical compound of seeds and roots of several plants of the *Apiaceae* family.[26] β-Bisabolene is found in several EOs from plants and is the major component of the EOs from the leaves of *Duguetia gardneriana*.[27] This compound exhibits antitumor properties[27] and is being tested for the treatment of breast cancer.[28] Elemicin is a phenylpropene compound found in plant EOs, especially elemi (*Canarium luzonicum*) from which the name derives.[29] This compound causes anticholinergic effects,[30] but the toxicology data available are lacking and it is not possible to assess if the present intake of elemicin may represent a health risk.[31]

Up to now, carrot seed EOs is a common fragrance component in cosmetics, perfumes and different categories of food products.[30] In the last case, their use is approved by the Food and Drug Administration (FDA, Code of Federal Regulations, Title 21, Volume 3, 21CFR182.20). The EOs of *D. carota* subsp. *maximus* possess another important desired characteristics represented by their antibacterial activity useful in biopreservation strategies. This information deepens the knowledge on the positive characteristics of the EOs extracted from this species; recently, *Valente et al.*[11] reported their antifungal and anti-inflammatory properties.

Conclusions

The EOs of *D. carota* subsp. *maximus* were highly effective against the bacteria used as indicators, both in terms of percentage of strains inhibited and width of the clear area. This behaviour was more evident against the *Gram*-positive bacteria rather than the *Gram*-negative species, confirming a common finding for the activity of the EOs, since the less sensitivity of the *Gram*-negative bacteria is due to the presence of the outer membrane that provides a strong impermeable barrier.[32] *MIC* Determination showed that EOs were effective at low concentrations against the most sensible strains of *L. monocytogenes*, *S. warneri*, *B. cereus*, and *A. haemolyticus*, indicating a great potential for application as biopreservative in foods. The chemical analysis revealed that *D. carota* subsp. *maximus* EOs extracted from the green seeds are characterized by a consistent presence of carotol, β-bisabolene, and isoelemicin. Thus, these EOs are rich in oxygenated sesquiterpenes and sesquiterpene hydrocarbons. In order to apply *D. carota* subsp. *maximus* EOs *in situ*, works are being prepared to evaluate the minimal doses able to express the antibacterial properties in different food model systems.

Experimental Section

Plant Material and Extraction of EOs

The plants of *D. carota* subsp. *maximus*, growing wild in Pantelleria Island (Sicily, Italy), were collected from

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Campobello area (36°82′ N, 11°98′ E) during August 2014. The specimens of the plants were deposited with the Herbarium, Orto Botanico, Palermo, Italy (ten samples). EOs were extracted from the green seeds by steam distillation with a 60-l stainless steel extractor (*Cucuzza Inox Impianti S.A.S.*, Grammichele, Italy) for 12 h.

Microbial Strains

Several bacterial strains of food origin belonging to species (*L. monocytogenes*, *A. guillouiae*, *A. haemolyticus*, *B. cereus*, *Serratia grimesii*, *Hafnia halvei*, *Hafnia paralvei*, *Enterobacter ludwigii*, *Raoultella ornithinolytica*, *St. maltophilia*, *S. epidermidis*, *S. haemolyticus*, *S. warneri*) responsible for human diseases were used as indicators (sensitive organisms) to test the inhibitory properties of *D. carota* subsp. *maximus* EOs. The strains *Lactobacillus sakei* LMG 2313 and *Listeria innocua* 4202 were also included in this study, because they are reported as strains highly sensitive.[33][34] All *L. monocytogenes*, except the strain ATCC 19114, were provided from the culture collection of the Section of Hygiene, Department of Sciences for Health Promotion and Mother Child Care 'G. D'Alessandro' (Palermo, Italy), while the other bacteria belonged to the culture collection of the Agricultural Microbiology Unit of the Department of Agricultural and Forest Science – University of Palermo (Italy). All bacteria were subcultured in Brain Heart Infusion (BHI) broth (*Oxoid*, Milan, Italy) incubated at 37 °C for 24 h, with the exception of *Lb. sakei* LMG 2313 that was cultured in MRS (*Oxoid*) incubated at 30 °C for 24 h.

Determination of Antibacterial Activity

EOs of *D. carota* subsp. *maximus* were tested against a cell density of approximately 10^7 CFU/ml of each bacterial strain in BHI or MRS soft agar (0.7% *w/v*) applying the paper disc diffusion method of *Kelmanson et al.*[35] with the modification reported by *Militello et al.*[13] Sterile water was used as negative control, while streptomycin (10% *w/v*) represented the positive control. The inhibitory activity was evaluated after 24 h of incubation at 37 °C and was scored positive when a definite clear area was detected around the paper discs. The tests were performed in triplicate.

Minimum Inhibitory Concentration

The antibacterial activity against the most sensitive strains (showing the highest diameter of the inhibition halo) was measured as *MIC*, which represents the most common expression of EO antibacterial performances.[36] *MIC* is defined as the lowest concentration of an active compound inhibiting visible growth of test organisms.[37]

The EOs were serially diluted (dilution factor = 2) in acetone (*Sigma–Aldrich*, Milan, Italy). Each test tube, containing 990 ml of broth medium and 10 ml of EO dilution, was inoculated with approximately 10^6 CFU/ml of a given sensitive strain. Acetone alone was used as negative control.

Chemical Composition of the EOs

The chemical profile of the EOs from *D. carota* subsp. *maximus* green seeds was performed by gas chromatography (GC) and mass spectrometry (MS) technique. Chromatographic analyses were carried out on a GC/MS system consisting of a GC instrument (*Agilent 6890*; Palo Alto, CA, USA) and a mass selective detector (*Agilent 5975 c*; Santa Clara, CA, USA). The column set used comprised a fused silica capillary column *Carbowax* (30-m length, 0.25-mm internal diameter and 0.25-μm film thickness; *Supelco*, Milan, Italy). All analyses were performed injecting 1 μl of the EOs in the split ratio (1:50) mode at a temperature of 250 °C. GC/MS instrument operated at 70 eV in the EI mode over the *m/z* range 30 – 550. Helium carrier gas flow was at 1 ml/min and the temperature of the oven was programmed from 40 to 230 °C at 4 °C/min and then held isothermal for 50 min; the injector temperature and the transfer line were set at 250 °C. All measurements were carried out in triplicate. The identification of the chemical compounds was achieved by matching the fragmentation patterns of the experimental mass spectra with the commercial library NIST05. The relative proportions of the individual components were expressed as percent peak areas normalisation, with all relative response factors being taken as one.

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Author Contribution Statement

R. Gaglio conducted the antimicrobial assays and contributed to the comment of the results. *M. Barbera*

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conducted the chemical analysis. *A. Aleo* supervised the choice of the pathogens and provided useful indications during the execution of the microbiological analysis and *MIC* determination. *I. Lommatzsch* extracted the EOs. *T. La Mantia* provided the raw materials, performed the botanical recognition of *D. carota* subsp. *maximus*, and contributed to identify the strengthens and the critical points during work execution. *L. Settanni* ideated the study, directed the experimental phases and wrote the manuscript.

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