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(*E*)-Methylisoeugenol and Elemicin: Antibacterial Components of *Daucus carota* L. Essential Oil against *Campylobacter jejuni*

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The essential oil of wild *Daucus carota* L. obtained from aerial parts at the end of the flowering stage (DCEO) was reported as antimicrobial against the human enteropathogen *Campylobacter jejuni*. The aim of the present study was to extend this analysis to other *Campylobacter* species and to identify the active compounds of the essential oil, subjected to GC, GC-MS, and ^{13}C NMR analysis. A minimum inhibitory concentration assay was used to quantify the antimicrobial activity of DCEO and the major components, isolated on column chromatography. Growth of all the *C. jejuni*, *Campylobacter coli*, and *Campylobacter lari* strains tested, including one multidrug resistant *C. jejuni*, was inhibited to the same extent by DCEO. Molecules that were responsible for the antibacterial activity were identified as (*E*)-methylisoeugenol and elemicin. Moreover, the use of structural analogues of these compounds allowed us to identify important features that may account for the activity.

KEYWORDS: Antibacterial activity; essential oil; *Daucus carota* L.; *Campylobacter jejuni*; *Campylobacter coli*; *Campylobacter lari*; multidrug resistance; (*E*)-methylisoeugenol; elemicin

INTRODUCTION

Essential oils are mixtures of naturally occurring substances showing various biological activities. Numerous oils have demonstrated antioxidative, antifungal, or anti-inflammatory effects (*1*, *2*). Some oils were described as antitumoral (*3*). However, the main biological activity exerted by essential oils was antibacterial activity (*4*).

During the last two decades, *Campylobacter* became one of the major causative agents of bacterial gastroenteritis in humans worldwide (*5*). In industrialized countries, the most frequently encountered *Campylobacter* species isolated from stool are *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari*. In addition to causing gastroenteritis, *Campylobacter* is also recognized as the most identifiable infection preceding Guillan−Barré syndrome, which leads to immune-mediated disorder and acute flaccid paralysis (*6*). *Campylobacter* is a zoonotic pathogen that colonizes a variety of wild and domestic animals. Commercial poultry are considered as the main reservoir of human *Campylobacter* infections (*7*), even though other risk factors for infection such as raw milk, water, and the direct zoonotic transmission from contact with infected pets (*8*, *9*) have been identified. Since most sporadic cases of human campylobacteriosis have been associated with the consumption of or contact with raw or undercooked poultry (*10*), the reduction of pathogens at relevant points of the "farm to fork" continuum could potentially reduce the risks to human health. Some processes or treatments were proposed to decrease or eliminate the population of *Campylobacter* on raw poultry meat by the use of carcass washer systems with chlorinated water, chemicals such as trisodium phosphate, or irradiation. One can also consider acting upstream by decreasing the *Campylobacter* carriage in the flocks to decrease the prevalence of campylobacteriosis (*11*). As antibiotic use in animal feeding as a growth promoter or preventive agent is becoming a major concern (*12*), there is a need for new natural antibacterial molecules to deal with food-borne pathogens.

Extracts of wild *Daucus carota* L., a plant belonging to the family of *Umbelliferae* (*Apiaceae*), were known to be antioxidative (*13*, *14*) and iron-chelative (*13*). Molecules from root extracts showed low antibacterial activities against *Staphylococcus aureus*, *Streptomyces scabies*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli* and antifungal activities against *Fusarium oxysporum* and *Aspergil-*

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Table 1. Characteristics of Strains Used in This Study and Susceptibility to *D. carota* L. Essential Oil

strains	species	MIC (μg/mL)	origin	source
CIP 103726	*C. jejuni*	250	human	CIP[a]
NCTC 11168	*C. jejuni*	250	human	reference strain (*29*)
CIP 103753	*C. coli*	250	human	CIP
CIP 70.54	*C. coli*	250	pig	CIP
CIP 102722T	*C. lari*	250	bird	CIP
F38011	*C. jejuni*	250	human	ref *20*
LV7	*C. jejuni*	250	human	LV[b]
LV9	*C. jejuni*	250	human	ref *30*
LV11	*C. jejuni*	500	human	LV
LM7a	*C. jejuni*	250	poultry	ref *31*
Lme27a	*C. jejuni*	125	poultry	ref *31*
99T403	*C. jejuni*	250	human	ref *30*

[a] CIP: Institut Pasteur Collection (Paris, France). [b] LV : Laboratoire Vialle (Bastia, France).

lus niger (*15*). Molecules from methanol seed extracts showed antibacterial activity against *S. aureus*, *E. coli*, *B. cereus*, *Citrobacter freundii*, and *Lactobacillus plantarum* (*14*). The antibacterial activity of the DCEO was tested against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *Candida albicans*, and *Penicillium expansum*, showing a sensibility of Gram-positive bacteria, whereas *P. expansum* and *P. aeruginosa* were not sensitive (*16*). The composition of the *D. carota* L. essential oil was variable according to the area of harvest, the part of the plant, and the stage of development (*17*−*19*). However, it could be summarized from literature data that leaf, stem, and blooming umbel oils are dominated by monoterpenes (*17*, *19*) or sesquiterpenes (*18*). Conversely, oils isolated from umbels in nest or seeds were dominated by β-bisabolene and β-asarone (*18*) or by (*E*)-methylisoeugenol accompanied by α-pinene and elemicin such as in commercial oils from Corsica, isolated from aerial parts harvested at the end of the flowering stage (*17*).

In a previous study, we observed that wild DCEO from Corsica was able to inhibit the growth of *C. jejuni* (*20*). Interestingly, none of the compounds of our essential oil was described as an antibacterial against *C. jejuni*. The aim of the present study was to extend this analysis to other *Campylobacter* species and to identify the compounds involved in this activity. Moreover, by comparison with chemical analogues to the identified active compounds, we were able to determine the structural features leading to this activity.

MATERIALS AND METHODS

Essential Oil Isolation. *D. carota* L. spp. *carota* essential oil (DCEO) was purchased from Huiles Essentielles et Hydrolats de Corse (Mandriolu, Corsica, France). The commercial oil was obtained by vapor distillation (6 h) with an industrial apparatus from air-dried wild *D. carota* L. aerial parts, collected at the end of the flowering stage. The essential oil yield was 0.6% (v/w). The isolated essential oil was filtered (0.22 μm filters) and stored at 4 °C until its analysis or its use in bioassays.

Chemicals. Allylbenzene, 4-allylanisole, methyleugenol (4-allyl-1,2-dimethoxybenzene), eugenol, isoeugenol, and 3,4-dimethoxystyrene were purchased from Sigma-Aldrich (L'Isle d'Abeau, France).

Bacterial Strains and Growth Conditions. The strains used in this work are listed in **Table 1**. *Campylobacter* strains were routinely grown at 37 °C on Muller Hinton agar (bioMérieux, Marcy l'Etoile, France) supplemented with 5% sheep's blood and Campylosel (bioMérieux) under microaerobic conditions obtained with GENbag (bioMérieux) for 24 h.

Chromatographic Fractionation. The bulk oil (5 g) was fractioned by column chromatography on silica gel (ICN 200−500 m, 100 g) and eluted with pentane *n*-C_5H_{12} and diethyl ether Et_2O. An apolar fraction F1 (2.8 g) and an oxygenated fraction F2 (Et_2O, 2.1 g) were obtained. The fraction F2 was further fractioned by column chromatography (ICN 63−200 μm, 350 g). Ten sub-fractions (F2.1−F2.10) were eluted with a gradient of solvents of increasing polarity (*n*-C_5H_{12}/Et_2O, 95:5 to 0:100). The fractions F2.4 and F2.9 were rechromatographed on a silica gel column (ICN 63−200 μm, 100 g) leading to two fractions (F2.4.1 and F2.9.1) containing (*E*)-methylisoeugenol (97%) and elemicin (98%), respectively.

GC Analysis. GC analyses were carried out using a PerkinElmer Autosystem GC apparatus equipped with a single injector and two flame ionization detectors (FID). The apparatus was used for simultaneous sampling to two fused-silica capillary columns (60 m × 0.22 mm i.d., film thickness 0.25 μm) with different stationary phases: Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). The oven temperature program was from 60 to 230 °C at 2 °C/min and then was held isothermally (30 min). Carrier gas: helium (1 mL/min). Injector and detector temperatures were held at 280 °C. Split injection was conducted with a ratio split of 1:80. Injected volume was 0.1 μL. The relative percentage of the oil constituents was calculated from the GC peak areas without using correction factors.

GC-MS Analysis. The oil and all fractions were analyzed with a PerkinElmer TurboMass detector (quadrupole), directly coupled to a PerkinElmer Autosystem XL equipped with two fused-silica capillary columns (60 m × 0.22 mm i.d., film thickness 0.25 μm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Other GC conditions were the same as described under GC Analysis. The ion source temperature was 150 °C, the energy ionization was 70 eV, and the electron ionization (EI) mass spectra were acquired over the mass range of 35−350 Da. The oil injected volume was 0.1 μL, and the fraction injected volume was 0.2 μL.

^{13}C NMR Analysis. The essential oil was analyzed by ^{13}C NMR on a Bruker Advance 400 Fourier transform spectrometer operating at 100.13 MHz, equipped with a 5 mm probe, in deuterated chloroform, with all shifts referred to internal tetramethylsilane (TMS). The ^{13}C NMR spectrum was recorded with the following parameters: pulse width was 4 μs (flip angle 45°), acquisition time was 2.7 s for the 128 K data table with a spectral width of 25 000 Hz (250 ppm), 3000 scans were accumulated, there was CPD mode decoupling, and the digital resolution was 0.183 Hz/point.

Component Identification. The identification of components was carried out by comparison of their mass spectra with those compiled in our laboratory-built spectral library, as well as by comparison of their retention indices with those of authentic samples or literature data (*21*, *22*). The GC retention indices (RI) on the polar and non-polar columns were determined relative to the retention time of a series of *n*-alkanes with linear interpolation. Identification of the major components was ensured by ^{13}C NMR, following a procedure developed and computerized in our laboratories and based on the comparison of ^{13}C NMR chemical shifts of components in the mixture with those of pure compounds compiled in a laboratory-built library (*17*).

Determination of Minimum Inhibitory Concentration (MIC). The MIC assays were performed by standard dilution methods (*23*). The MIC was defined as the lowest product concentration that prevented visible growth of bacteria. All tests were performed on Muller Hinton agar. Briefly, 30 μL of 2-fold serial dilutions in DMSO (dimethylsulfoxyde, Sigma-Aldrich, L'Isle d'Abeau, France) was added to 15 mL of agar to obtain concentrations ranging from 30 to 1000 μg/mL tested product. The resulting agar solutions were mixed at high speed for 15 s, immediately poured into sterile Petri dishes, and then allowed to set for 30 min. The plates were then spot inoculated by pipetting 10^5 CFU of the desired strain on the spot on the plates. A negative control was prepared without essential oil, using only DMSO. Gentamycin and erythromycin (Sigma-Aldrich) were used as positive controls. Inoculated plates were incubated at 37 °C for 24 h. After the incubation period, the plates were observed and recorded for the presence or absence of growth. Each test was repeated at least 3 times.

RESULTS AND DISCUSSION

Chemical Composition and Screening of Biological Activity. Chemical analysis of the commercial oil sample led to the

Table 2. Composition of *D. carota* L. Essential Oil[a]

constituents	RIa	RIp	%	NMR[b]
α-pinene	929	1013	15.9	^{13}C NMR
camphene	943	1055	0.7	^{13}C NMR
sabinene	963	1120	2.7	^{13}C NMR
β-pinene	969	1110	1.1	^{13}C NMR
myrcene	979	1159	2.0	^{13}C NMR
p-cymene	1011	1250	0.2	
limonene	1020	1183	1.7	^{13}C NMR
(*Z*)-*β*-ocimene	1024	1230	0.1	
γ-terpinene	1047	1227	0.2	
terpinen-4-ol	1161	1600	0.2	
bornyl acetate	1269	1575	0.5	^{13}C NMR
methyleugenol	1367	2009	0.2	
α-cedrene	1417	1562	0.3	
β-cedrene	1425	1594	0.2	
trans-α-*bergamotene*	1432	1580	0.2	
veratraldehyde	1434	2400	0.2	
(*E*)-*β*-farnesene	1446	1663	0.5	^{13}C NMR
(*E*)-methylisoeugenol	1465	2188	21.8	^{13}C NMR
germacrene D	1479	1706	0.5	
6-*epi* shyobunone	1480	1854	0.5	^{13}C NMR
β-selinene	1485	1716	0.5	^{13}C NMR
α-selinene	1494	1721	0.9	^{13}C NMR
β-bisabolene*	1502	1725	21.3	^{13}C NMR
shyobunone*	1502	1900	1.3	^{13}C NMR
elemicin	1518	2232	16.3	^{13}C NMR
(*E*)-α-bisabolene	1533	1772	1.1	^{13}C NMR
11α-*H*-himachal-4-en-1*β*-ol	1638	2110	0.7	^{13}C NMR
(*E*)-asarone	1642	2463	0.8	^{13}C NMR
α-bisabolol	1672	2217	0.2	
eudesm-7(11)-en-4α-ol	1682	2300	0.7	^{13}C NMR
isocalamendiol	1728	2500	0.2	
total			93.7	

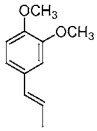
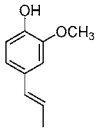
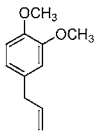
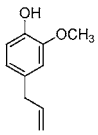
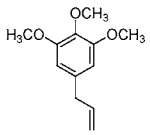
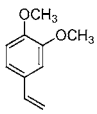
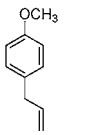
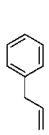
	%
monoterpene hydrocarbons	24.6
sesquiterpene hydrocarbons	25.5
oxygenated monoterpenes	0.7
oxygenated sesquiterpenes	3.6
phenyl propanoids	39.1
other	0.2

[a] Order of elution and percentages of components were given on the RTX-1 column, except for compounds with an asterisk, which were percentage on the polar column. RIa and RIp: retention indices on RTX-1 apolar column and RTX-Wax polar column, respectively. All compounds have been identified by GC (RI) and GC-MS. [b] Compounds also identified by ^{13}C NMR.

identification of 31 components that represented 93.7% of the total amount (**Table 2**). The major components were (*E*)-methylisoeugenol (21.8%), *β*-bisabolene (21.3%), elemicin (16.3%), and α-pinene (15.9%). To the best of our knowledge, none of these compounds was already described as effective against *C. jejuni* growth.

We previously showed the susceptibility of *C. jejuni* for wild DCEO by testing the antibacterial activity of that oil against a clinical isolate of *C. jejuni*, the F38O11 strain (MIC value of 250 μg/mL) (*20*). Herein, we demonstrated that the *C. jejuni* reference strain and other bacteria belonging to the genus *Campylobacter* such as *C. coli* and *C. lari* exhibited the same level of susceptibility with a MIC value of 250 μg/mL (**Table 1**). In comparaison, the MIC values of gentamycin and erythromycin against the NCTC 11168 strain were, respectively, 0.125 and 0.5 μg/mL. Four clinical isolates were also explored for their susceptibility. Three of them, F38O11, LV7, and LV9, showed the same level of susceptibility, with a MIC value of 250 μg/mL. By contrast, the human isolate LV11 appeared to be less sensitive with a MIC value of 500 μg/mL. *C. jejuni* isolated from poultry (LM7A and Lme27A) were also susceptible with MIC values of 250 and 125 μg/mL. Patients usually recover from *Campylobacter* infections without requiring antibiotic treatment, but in cases of severe illness or persistent enteritis, erythromycin or fluoroquinolones are the antibiotics of choice. The rise of antibiotic resistance in *C. jejuni* (*24*) calls for new molecules effective against resistant *C. jejuni*. We tested the activity of DCEO against a multidrug resistant strain (99T403). The growth was inhibited at the same level as compared to non-resistant strains (**Table 1**).

Table 3. Structure and Activity against *C. jejuni* of Various Phenyl Propanoids and Dimethoxystyrene

Components	(E)-methylisoeugenol 	Isoeugenol 	
MIC (μg/mL)	125	125	
Components	Methyleugenol 	Eugenol 	Elemicin 
MIC (μg/mL)	250	250	250
components	3,4-dimethoxystyrene 	Allylanisole 	Allylbenzene 
MIC (μg/mL)	250	1000	> 1000

Identification of Active Compounds. The essential oil was fractionated into apolar (F1) and polar (F2) fractions (see Materials and Methods) and tested for antibacterial activity against the NCTC 11168 reference strain. Fraction F1, containing hydrocarbons, was not active (MIC >1000 μg/mL), while a strong biological activity was obtained with fraction F2 (MIC = 250 μg/mL). The polar fraction was characterized by the presence of (*E*)-methylisoeugenol (41.8%) and elemicin (33.6%) The percentages of other components, except shyobunone (3.4%), were smaller than 2%. To verify if these phenyl propanoids were responsible for the antimicrobial activity, the oxygenated fraction was submitted to repeated chromatography, and two sub-fractions that contained (*E*)-methylisoeugenol (97%) and elemicin (98%), respectively, were tested against the NCTC 11168 strain. (*E*)-Methylisoeugenol inhibited growth (MIC = 125 μg/mL), while elemicin inhibited the growth but to a lesser extent (MIC = 250 μg/mL). The activities of the two sub-fractions were also tested against F38011 and MDR 99T403 strains leading to the same results.

Structural Features Involved in the Biological Activity. Both (*E*)-methylisoeugenol and elemicin are phenylpropanoids bearing oxygenated functions on the aromatic ring and a double bond on the side chain. To evaluate the structural features involved in the biological activity, we compared the antimicrobial activity of (*E*)-methylisoeugenol and elemicin with commercial molecules belonging to the same family against the NCTC 11168 strain (**Table 3**). Allylbenzene, 4-allylanisole, methyleugenol (4-allyl-1,2-dimethoxybenzene), eugenol, isoeugenol, and 3,4-dimethoxystyrene were tested.

First, no activity was observed with allylbenzene (MIC > 1000 μg/mL) or allylanisole (MIC = 1000 μg/mL), in which none or only one oxygenated function on the aromatic ring was contained in their structure. Obviously, two oxygenated functions are needed to induce an antibacterial activity.

(*E*)-Methylisoeugenol and (*E*)-isoeugenol on one hand and (*E*)-methyleugenol and eugenol on the other hand exhibited the same MIC (125 and 250 μg/mL, respectively). Consequently, the nature of the oxygenated function (hydroxyl or methoxy group) had no influence on the antibacterial activity against *C. jejuni*.

Conversely, the position of the double bond on the side chain plays an important role. Indeed, (*E*)-methylisoeugenol and (*E*)-isoeugenol, bearing the propenyl sub-structure, are more active (MIC = 125 μg/mL) than (*E*)-methyleugenol and eugenol itself, bearing the allyl sub-structure (MIC = 250 μg/mL). Friedman et al. (*25*) have previously reported that eugenol and isoeugenol were active against *C. jejuni*, but they showed that eugenol was more active than isoeugenol.

It should be mentioned that the replacement of the propenyl side chain by a vinyl group in 3,4-dimethoxystyrene, for instance, led to a decrease of the antimicrobial activity (MIC = 250 g/mL instead of 125 μg/mL). Finally, it appears, by comparing the activity of (*E*)-methyleugenol and elemicin, that the presence of a third methoxy group had no influence on the antibacterial activity (MIC = 250 g/mL).

(*E*)-Methylisoeugenol and elemicin appeared to be a potential response to *Campylobacter* infections. De Vicenzi et al. (*26*) have reported that the genotoxic potential of elemicin was uncertain. Hasheminejad and Caldwell (*27*) have described that methylisoeugenol was non-genotoxic. Moreover, a 28 day feeding study with methylisoeugenol in rats did not demonstrated adverse effects (*28*).

(*E*)-Methylisoeugenol and elemicin could be used to decrease the *Campylobacter* carriage in poultry flocks by using them as food additives. (*E*)-Methylisoeugenol and elemicin could be also used as a base to develop new therapeutic agents against MDR *C. jejuni*. But, the data about the toxicity of these two components have to be developed, and the efficiency in vivo must now be tested.

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