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SHORT COMMUNICATION

Susceptibility of *Helicobacter pylori* to Essential Oil of *Dittrichia viscosa* subsp. *revoluta*

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The essential oil of *Dittrichia viscosa* subsp. *revoluta* and its fractions were assessed for anti-*Helicobacter* activity. The essential oil was isolated by hydrodistillation, submitted to flash column chromatography and analysed by gas chromatography, gas-chromatography coupled to mass spectrometry and ^{13}C-nuclear magnetic resonance. The anti-*Helicobacter* activity was determined by incorporation of the crude essential oil and oxygenated fractions of the oil into the culture medium. At a concentration of 0.025 μL/mL no recovery was registered when one of the oxygenated fractions of the oil, mainly constituted by 3-methoxy cuminyl isobutyrate (about 40%), was used. This fraction revealed a higher activity against the six *H. pylori* strains tested when compared with the other oxygenated fractions. The crude essential oil at a concentration of 0.33 μL/mL reduced the initial population of *H. pylori* CCUG 15818 of 8.52 ± 0.30 $\log_{10}$ cfu/mL to 7.67 ± 0.22 $\log_{10}$ cfu/mL. The susceptibility of several *Helicobacter pylori* strains to the oxygenated fraction of *Dittrichia viscosa* subsp. *revoluta* essential oil suggests the possible use of these natural products in combating this widespread infection.

Keywords: Helicobacter pylori; *Dittrichia viscosa*; essential oil; antibacterial activity.

INTRODUCTION

Helicobacter pylori is a Gram-negative spiral-shaped bacterium that colonizes the human stomach and duodenum, despite the relative inhospitable gastric environment to the majority of bacteria (Lamarque and Peek Jr, 2003; Stoicov *et al.*, 2004). Chronic gastric infection by *H. pylori* may originate various gastric-related diseases such as chronic gastritis, peptic ulceration and gastric cancer. Most colonized individuals (approximately 80%) remain asymptomatic, presenting mild but diffuse inflammation of the stomach, showing little or no atrophy throughout their lifetimes indicating that the bacterium and the host adapt to each other through a long-term equilibrium (Blaser and Atherton, 2004; Joseph and Kirschner, 2004). On the other hand, there are those individuals that are chronically infected and develop severe disease such as adenocarcinoma (Joseph and Kirschner, 2004).

A combination of therapeutic agents has been used in the eradication of *H. pylori*: (a) triple therapy with the antibiotics metronidazole, tetracycline or amoxicillin and bismuth; (b) double therapy constituted by antibiotics jointly with proton-pump inhibitors (omeprazole) or with H_2-blockers (ranitidine). The resistance to metronidazole, in about 20% of patients especially those already treated with metronidazole, and the high side effect profile (headache, dizziness, nausea, diarrhea, pseudomembraneous colitis, mycosis, sore mouth and tongue, drug hypersensitivity and paraesthesia) are real problems of the triple therapy. The results of the double therapy on *H. pylori* eradication are also inconsistent (Pakodi *et al.*, 2000). Along with the development of resistance to antibiotics there are other factors that contribute to therapeutic failure: cost and efficacy of antibiotics regarding the pH (for instance, amoxicillin is most active at a neutral pH and tetracycline has greater activity at a low pH (Wang and Huang, 2005).

Many naturally occurring compounds found in the dietary and medicinal plants, herbs and fruit extracts have been reported to possess antimicrobial activities (Lin *et al.*, 2005; Li *et al.*, 2005; Wang and Huang, 2005; Chun *et al.*, 2005). For instance, recent research has demonstrated that resveratrol produced during wine fermentation is active in acidic conditions (e.g. in stomach) and may be linked to inhibition of *H. pylori* (Lin *et al.*, 2005). Green tea catechins (epigallocatechin and epicatechin) have been shown to inhibit the growth of *H. pylori* in a guinea-pig model of infection (Stoicov *et al.*, 2004). In some cases, flavonoids and isoflavonoids present in ethanol extracts of some plants were revealed to possess potent anti-*H. pylori* activities (Fukai *et al.*, 2002; Li *et al.*, 2005). Generally, flavonoids have a range of biological activities and pharmacological effects, including also a pronounced antiulcerogenic activity (Motilva *et al.*, 1992). The activity of essential oils against *Helicobacter pylori* was reported (Ohno *et al.*, 2003) and amongst the 13 essential oils tested for *H. pylori* growth inhibition, *Cymbopogon citratus* (lemongrass) and *Lippia citriodora* (lemon verbena) were demonstrated to have the highest bactericidal activities even

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at low pH values (pH 4.0 and 5.0). During another investigation the combination of 0.0312 mg/mL of origanum and monolaurin was cidal for *H. pylori* strain ATCC 49503 (Preuss *et al.*, 2005).

Dittrichia viscosa W. Greuter Asteraceae is a subshrub widespread in Europe, especially in Mediterranean areas (Devesa, 1987). This species has been used for years in folk medicine in the treatment of gastroduodenal diseases (Font Quer, 1978). Some studies revealed that the antiulcerogenic effect of *D. viscosa* is mainly due to its flavonoid fraction (Grande *et al.*, 1992; Martín *et al.*, 1988). In addition to the flavonoid fraction, other authors studied the volatile oil of *D. viscosa* collected in Turkey and Spain (Pérez-Alonso *et al.*, 1996; Camacho *et al.*, 2000). The essential oil of *D. viscosa* subsp. *viscosa* collected in Portugal was also subjected to studies in order to elucidate its ability to prevent *H. pylori* growth (Silva *et al.*, 2005). The investigators concluded that the chemical composition of the oil isolated from plants collected in Portugal was closer to that previously reported (Camacho *et al.*, 2000) for the oil obtained from plants from Spain (Province of Jaénz) and for that from Corsica (Blanc *et al.*, 2006), mainly in the presence of the major components fokienol and (*E*)-nerolidol. Moreover in that study, a small amount of essential oil of *D. viscosa* subsp. *viscosa* from Portugal was detected, for the first time, which could drastically reduce the growth of *H. pylori* (Silva *et al.*, 2005).

The main goal of the present contribution was to determine the ability of the essential oil of the Portuguese endemic *D. viscosa* subsp. *revoluta* and some of its fractions to inhibit the growth of *H. pylori*.

METHODS

Plant material. The aerial parts of *Dittrichia viscosa* subsp. *revoluta* were collected in the region of Algarve, Portugal in the flowering phase, during July–September 2002.

Isolation procedure. An essential oil sample was isolated by water distillation for 4 h from fresh material, using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Anonymous, 1996). The essential oil was stored at 4 °C in the dark prior to analysis. The yield of the essential oil was 0.3% (v/w).

Oil fractionation. The bulk oil (2 g) was submitted to flash chromatography (FC), silica gel 63–200 μm. The first two fractions (F) were eluted with pentane (F1 = 26 mg; F2 = 133 mg); two fractions were eluted with pentane/diethyl oxide (95/5) (F3 = 48 mg; F4 = 134 mg); two fractions were eluted with pentane/diethyl oxide (75/25) (F5 = 459 mg; F6 = 443 mg); the last two fractions were eluted with diethyl oxide (F7 = 339 mg; F8 = 88 mg).

Gas chromatography. Analytical GC was carried out in a Perkin-Elmer Autosystem XL gas chromatograph apparatus with dual FID and fused-silica capillary columns with different stationary phases: BP-1 (polymethylsiloxane 50 m × 0.22 mm i.d., film thickness 0.25 μm), and BP-20 (polyethylene glycol 50 m × 0.22 mm i.d., film thickness 0.25 μm). Carrier gas: helium at 0.8 mL/min; splitting ratio: 1:60; injection temperature: 250 °C; oven temperature programmed from 60 to 220 °C at 2 °C/min and then held isothermal (20 min); detector temperature: 250 °C.

Gas chromatography-mass spectrometry. Analyses were carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.20 μm), interfaced with a Hewlett-Packard mass-selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described earlier; interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scan/s: 4.51.

^{13}C-NMR. ^{13}C-NMR spectra of the crude oil as well as the fractions of flash chromatography were recorded on a Bruker AC200 Fourier Transform spectrometer operating at 50.323 MHz, equipped with a 10 mm probe (200 mg oil; 2 mL $CDCl_3$; 5000 scans) or 5 mm probe (70 mg oil; 0.5 mL $CDCl_3$; 10 000 scans), with all shifts referred to tetramethylsilane (TMS). ^{13}C spectra were recorded with the following parameters: pulse width (PW), 5 μs [or 3 μs] (flip angle 45°); acquisition time, 1.3 s and relaxation delay (Dl), 2 s (total recycling time, 3.3 s) for 32 K data table with a spectral width (SW) of 12 500 Hz (250 ppm); CPD mode decoupling; digital resolution, 0.763 Hz/pt. An exponential multiplication of the free induction decay with the line broadening of 1.0 Hz was applied before Fourier transformation.

Qualitative and quantitative analyses. The identity of the components was achieved from their retention indices on polar and apolar columns, determined relative to the retention times of a series of C_8-C_{22} *n*-alkanes with linear interpolation with those of authentic components included in our own laboratory database. Acquired mass spectra were compared with reference spectra from our own library or from literature data (Adams, 2004; Joulain and Konig, 1998). The identity was also determined by ^{13}C-NMR spectroscopy, following the methodology developed and computerized in our laboratories (Tomi *et al.*, 1995).

Relative amounts of individual components were calculated based on GC peak areas without flame ionization detector (FID) response factor correction.

Antibacterial determination. Crude essential oil and oxygenated fractions of the oil were eluted in 2-propanol (10%, v/v) and all concentrations were from the same stock solution of the eluted essential oil. Essential oil and the oxygenated fractions of the oil were incorporated into the Columbia agar medium (supplemented with 10% blood, v/v). The 2-propanol was tested previously for antimicrobial activity and at the concentration used no effect on bacterial viability was registered. *Helicobacter pylori* strain 3, 28, 30, 40 and 147 are clinical isolates from gastric biopsies obtained at Faro Hospital (Portugal) and belong to the Microbiology Laboratory of Faculty of Natural Resources Engineering of University of Algarve. *H. pylori* strains CCUG 15818, 26695 and J99 were used as laboratory

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strains. Bacterial viability was determined by the drop method (Chen *et al.*, 2003). Briefly, the dilutions of the sample were done using a 96-well plate, initially 250 μL of the sample was distributed into the first well of each row of the microplate followed by the preparation of 10-fold serial dilutions using a multichannel pipette (Transferpette-8, Brandtech, USA) by transferring 20 μL from the first row into 180 μL of medium on the next column. The inocula were homogenized by pipetting at least 10 times and the followed dilutions were done by repeating the process having changed pipette tips between dilutions. Afterwards, six replicates of 10 μL from each of the six selected dilutions were distributed onto Columbia agar medium (supplemented with 10% blood, v/v) at appropriate essential oil concentration. Inocula were allowed to dry before to introduce them in anaerobic jars (Oxoid, Basingstoke, Hampshire, UK) in the presence of Anaerocult A pack for generation of anaerobic conditions. The plates were incubated during 5–7 days at 37 °C.

The MIC of the antibiotic amoxicillin was determined by the ε-test (AB Biodisk, Sweden) in Columbia medium supplemented with blood (10%, v/v).

RESULTS AND DISCUSSION

The identified compounds in the essential oil of *D. viscosa* subsp. *revoluta* are indicated in Table 1, where the components are listed in order of their elution on the BP-1 column. The essential oil of *D. viscosa* subsp. *revoluta* was mainly constituted by oxygenated compounds. The fractions eluted with pentane/diethyl oxide (75/25), that is, fraction F5 and F6 provided 459 and 443 mg, respectively; the elution with diethyl oxide produced fractions F7 and F8 with a total mass of 339 and 88 mg, respectively. The major components present in the oil were 3-methoxy cuminyl isobutyrate (12%), α-cadinol (6.3%), eudesm-6-en-4α-ol (4.8%) and δ-cadinene (4.6%). The presence of 3-methoxy cuminyl isobutyrate was previously reported by Ascensão *et al.* (1999) in the oil of *D. viscosa* subsp. *revoluta* representing 15% of the total oil of the inflorescences. The presence of costic acid, isocostic acid and 4-en-ilicic acid were reported by Blanc *et al.* (2006, 2005, 2004) for *D. viscosa* ssp. *viscosa* and *Inula graveolens* from Corsica. The direct quantification of these three

Table 1. Composition of the essential oil of *D. viscosa* subsp. *revoluta*. Components listed according to their elution on the BP1 column

Component	RI[a]	RI[b]	Percentage in sample
1,8-Cineole	1021	1211	0.5
Linalool	1083	1543	0.4
p-Mentha1,5-dien-8ol	1145	1722	0.4
Terpinen-4ol	1162	1599	0.2
p-Mentha1(7),2-dien-8-ol	1169	1780	0.5
Bornyl acetate	1269	1577	0.5
α-Cubebene	1349	1453	0.1
α-Ylangene	1372	1479	0.4
α-Copaene	1377	1488	0.7
α-Gurjunene	1411	1525	0.3
trans-Caryophyllene	1419	1593	1.0
β-Copaene	1426	1586	0.1
allo-Aromadendrene	1459	1640	0.4
γ-Muurolene	1471	1683	0.7
α-Amorphene	1475	1683	0.8
Bicyclosesquiphellandrene	1488	nd	0.5
α-Muurolene	1494	1719	1.4
γ-Cadinene	1508	1756	2.4
cis-Calamenene+*trans*-Calamenene	1511	1832	0.9
δ-Cadinene	1517	1756	4.6
α-Cadinene	1530	1789	0.4
Caryophyllene oxide	1573	1984	1.1
Eudesm-6-en-4α-ol	1608	2165	4.8
γ-Eudesmol	1622	2171	1.1
T-Cadinol	1630	2171	2.1
T-Muurolol	1630	2186	2.7
Caryophylla-4(14),8(15)-diene-5α-ol	1630	2298	1.1
α-Eudesmol	1643	2220	1.4
α-Cadinol	1643	2233	6.3
Cadalene	1656	2224	0.2
3-Methoxy cuminyl isobutyrate	1682	2271	12.0
Pentacosane	nd	2499	0.2
Isocostic acid	nd	nd	
Costic acid	nd	nd	
4-en-Ilicic acid	nd	nd	

[a] RI: retention indices measured on apolar column (BP-1).
[b] RI: retention indices measured on polar column (BP-20).
nd: not determined.

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eudesman-type acids by GC was not performed due to their relative low volatility, being necessary to proceed to a previous derivatization in order to transform them in the corresponding methyl esters prior to the GC analysis (Grande *et al.*, 1992; Blanc *et al.*, 2005). Regarding this, such acids were identified by ^{13}C-NMR. The fractions used for the determination of anti-*H. pylori* activity were those mainly composed of oxygenated compounds not only by their highest mass obtained but also by the oxygenated components that are generally responsible for biological activities, namely fraction 4, 5, 6 and 7. 3-Methoxy cuminyl isobutyrate was the major component detected in fraction F5 constituting about 40% of the fraction. In fraction 6, one component not identified constituted 25% of the total oil, followed by T-cadinol, T-muurolol and caryophylla-4(14),8(15)-diene-5α-ol that reached a percentage of 18% and finally eudesm-6-en-4α-ol (10%). The fraction F7 was mainly constituted by α-cadinol and α-eudesmol with a total percentage of 31%. The olefinic fractions F1 and F2 eluted by pentane were mainly composed of α-copaene (34%), γ-cadinene (19%), α-ylangene (15%), α-gurjunene (14%), α-muurolene (10%) and δ-cadinene (10%).

Crude essential oil of *Dittrichia viscosa* subsp. *revoluta* demonstrated to have anti-*Helicobacter* activity determined against the laboratory strain CCUG 15 818. At a concentration of 0.33 µL/mL the initial population of 8.52 ± 0.30 $\log_{10}$ cfu/mL was reduced to 7.67 ± 0.22 $\log_{10}$ cfu/mL.

Susceptibility of *H. pylori* strain CCUG 15 818 to all oxygenated fractions tested; F4, F5, F6 and F7 was observed. However, fraction 5, mainly constituted of 3-methoxy cuminyl isobutyrate, showed the highest activity against the seven strains used, followed by fraction F7 mainly composed of α-cadinol and α-eudesmol. Anti-*Helicobacter* activity of the two active fractions is represented in Fig. 1. *H. pylori* strain CCUG 15 818 was the most susceptible; at a concentration of 0.025 µL/mL no growth was registered. *H. pylori* strains J99 and strain 3 were middle resistant. At a concentration of 0.03 µL/mL the initial population of strains J99 (9.61 ± 0.07 cfu/mL) and strain 3 (9.77 ± 0.28 $\log_{10}$ cfu/mL) were reduced 1 log. At the highest concentration no recovery of cells was registered. The most resistant strains were strain 26 695 and strain 147. At the highest concentration tested (0.035 µL/mL) the initial population of strain 26 695 (9.02 ± 0.76 $\log_{10}$ cfu/mL) was reduced 4 log, whereas the initial population of strain 147 (9.04 ± 0.07 $\log_{10}$ cfu/mL) was reduced 6 log. Fraction 7 demonstrated an anti-*Helicobacter pylori* activity only against the strain *H. pylori* CCUG 15 818 (Fig. 1). At a concentration of 0.025 µL/mL the initial population of this strain (9.01 ± 0.04 $\log_{10}$ cfu/mL) was reduced 5 log. The other strains did not experience a significant reduction (Fig. 1). Regarding the susceptibility of *H. pylori* strains to the antibiotic amoxicillin the MIC value for *H. pylori* strain J99, CCUG 15 818, 147, was 0.50 µg/mL, 0.16 µg/mL, 0.16 µg/mL and *H. pylori* strains 3, 28 and 40 were resistant to higher than 256 µg/mL. It is interesting to verify that the *H. pylori* strains 3 and 28 that are highly resistant to the antibiotic amoxicillin are susceptible to oxygenated fraction F5.

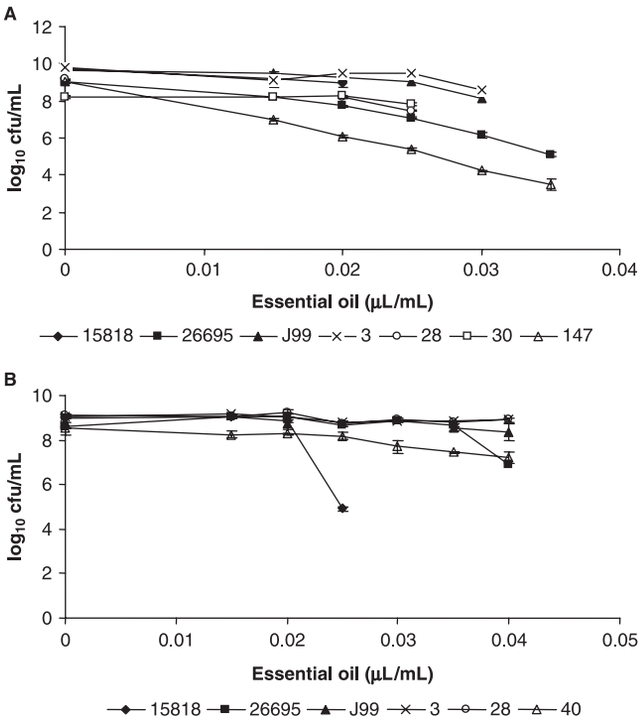

Figure 1. Susceptibility of *Helicobacter pylori* strains CCUG 15 818, 26 695, J99, 3, 28 and 40 to the oxygenated fractions 5 (A) and 7 (B) of the essential oil of *Dittrichia viscosa* subsp. *revoluta*. Data are the mean of three independent experiments. Bars represent the standard deviation.

In spite of chemical differences detected in both subspecies, our previous results on the determination of the antimicrobial activity of *Dittrichia viscosa* subsp. *viscosa* essential oil indicated a specific activity of this essential oil against *H. pylori* but a null activity against an important foodborne pathogen *Listeria monocytogenes* (Silva *et al.*, 2005). The use of the essential oil of *Dittrichia viscosa* subsp. *viscosa* and *D. viscosa* subsp. *revoluta* may greatly contribute to an efficient control of this bacterial pathogen that is spread amongst the world population and for which the mode of transmission is still controversial. Moreover due to the increase of the multiresistance pattern and also the increasing tendency of the public to consume 'green products' the use of these types of compounds will greatly help to combat this infection accompanied by consumer confidence and support which plays an important role to the therapy success.

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