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Evaluation of the antimicrobial and antioxidant activities of essential oils, extracts and their main components from oregano from Madeira Island, Portugal

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ABSTRACT

Origanum vulgare subsp. *virens* growing wild in Madeira Island, Portugal was studied within the ongoing investigations on polymorphic Lamiaceae species. The antimicrobial activity of the essential oils and *n*-hexane extracts of origanum was determined against 10 strains of bacteria and yeasts, found as human pathogenic or food spoilage microorganisms. The essential oils, *n*-hexane extracts and isolated compounds showed moderately activity, compared to standard antibiotics, inhibiting all tested bacteria except *Pseudomonas aeruginosa*. The most sensitive microorganism was *Mycobacterium smegmatis*, reaching MIC = 25 µg mL⁻¹. The results obtained suggest a potential application of these oils in preventing the human pathogenic and food spoilage due to microorganism's growth. The essential oils and *n*-hexane extracts have greater RSC than polar extracts, probably due to the high contents in thymol, which demonstrated the highest activity in the DPPH assay. All studied origanum samples showed a large content in non-esterified 1-hexacosanol, C₂₆H₅₄O, accumulated mainly in bracts and flowers.

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1. Introduction

Origanum vulgare L. subsp. *virens* (Hoffmanns. & Link) Letswaart (sometimes called the Moroccan origanum) is the common oregano that thrives naturally in almost every region of Portugal, especially on the edges of fields, on dry and uncultivated or waste ground. In Madeira Island, it is very common in the edges on paths in the Laurisilva forest, up to 1500 m of altitude.

The antimicrobial properties of volatile aromatic oils and medium-chain fatty acids derived from edible plants have been recognized since antiquity. Origanum oil, used as food-flavouring agent, possesses a broad spectrum of antimicrobial activity due, at least in part, to its high content of phenolic derivatives, such as carvacrol and thymol (Preuss et al., 2005). In the literature, there are many reports relating the chemical composition and the antimicrobial properties of the essential oils of various origanum species, and their application in various commercial preparations, as antimicrobials and antioxidants (Baydar, Sagdic, Ozkan, & Karadogan, 2004; Kulisic, Radonic, Katalinic, & Milos, 2004).

Earlier studies have demonstrated the ability of origanum oil to retard and to inhibit the growth of various food spoiling organisms including the species of *Aspergillus* (mycotoxinogenic filamentous fungi) and industrial yeasts. Origanum oil completely inhibited the growth of *Candida albicans* at 0.25 mg mL⁻¹, being the most potent of the essential oils tested and proved to be bactericidal in culture to two strains of *Staphylococcus aureus* at 0.25 mg mL⁻¹ (Salgueiro et al., 2003). However, the antimicrobial activity is strongly dependent on composition and origanum shows a wide variety of subspecies with various chemotypes within each of those, rendering it difficult to compare results from different bibliographic sources. According to Chorianopoulos et al. (2004), the antimicrobial activity of origanum essential oil depends greatly on the chemical composition in carvacrol and thymol.

The current trend adopted by food production, legislative offices and consumers has demanded a progressive retreat of chemical additives in food conservation systems, searching alternative substances in order to preserve final products from food spoilage and diseases caused by microorganisms and oxidation.

The present work was undertaken to determine the chemical composition of essential oils from origanum growing wild in several locations of Madeira Island, and to evaluate any relationships between composition and the radical scavenging capacity and the antimicrobial activity towards human pathogenic and food spoilage fungi and bacteria, in the search for alternative to commercial preservatives.

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2. Materials and methods

2.1. Plant material

O. vulgare subsp. *virens* samples were collected in four locations of Madeira Island, Portugal, between 500 and 1000 m of altitude. Samples are designated from 1 to 4, collected respectively at Calheta, Encumeada, Ponta do Sol and Santo da Serra. Aerial parts were dried in well ventilated spaces away from sun light. Vouchers were included on the collection of Madeira Botanical Gardens (MADJ306106, MADJ306206, MADJ306306, MADJ306406).

2.2. Essential oils isolation

Air-dried plant materials were submitted hydrodistillation according to European Pharmacopeia, using a Clevenger type apparatus. The essential oils amounts were determined gravimetrically.

2.3. Essential oils analysis

Chromatographic analysis was carried out on a Hewlett Packard 5890 series II GC equipped with an Automatic Sampler HP 6890 series injector linked to two injector modules, two flame ionization detectors and two columns, together with a computer station for data treatment. The columns were an OV-101 fused silica (50 m × 0.25 mm, 0.25 μm film thickness) and Supelcowax 10 (30 m × 0.25 mm, 0.25 μm film thickness). Oven temperature was held at 70 °C for 5 min and then programmed to 230 °C at a rate of 2 °C/min with a split ratio of 1:45. The detector and injector were kept at 250 °C and 240 °C, respectively. Pure helium was used as carrier gas, at constant pressure 55 kPa for the OV-101 and 45 kPa for the Supelcowax 10. A 1 μl of oil was injected. Identification of compounds was based on their mass spectra and their retention indices (RI) obtained from calculated values relative to C₈–C₂₀ *n*-alkanes. The intensity of each peak was integrated. Each sample was analysed three times. The average peak areas of all GC signals were determined and the percentage of each component peak was calculated by comparing its average area to the total area. The GC–MS analysis for identification of compounds was carried out in a Varian Saturn 3 GC–MS (Ion trap) operating in EI mode and using an HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness), carrier gas helium, constant pressure 90 kPa, split 1:20. The oven was programmed initially from 70 °C with 2 min hold up time to the final temperature of 230 °C with 5 °C/min ramp. The final temperature hold time was 5 min. The inlet and GC/MS interface temperatures were kept at 250 °C and 280 °C, respectively. The temperature of EI 70 eV source was 200 °C with full scan (25–450 *m/z*), scan time 0.3 s. The mass spectra of essential oil and extract components were identified by comparing the mass spectra of the analytes with those of authentic standards from the mass spectra of Wiley 6.0 and Mass Spectra Library (NIST 98), and with corresponding data of components from reference oils analysed in our laboratory.

2.4. Preparation of extracts

The air-dried powdered aerial parts were successively extracted at room temperature with *n*-hexane, chloroform, ethyl acetate and methanol. Chlorophylls were removed with activated charcoal and the solvents evaporated under reduced pressure, at room temperature. During the solvent evaporation of the *n*-hexane extracts of all plant materials, white crystals of melting point 77–78 °C were formed. These were filtered and characterized, being identified as pure *n*-hexacosanol (ceryl alcohol) by FTIR, mass spectrometry and NMR.

2.5. Antioxidant activity

Free Radical Scavenging Capacity (RSC). The RSC was evaluated by measuring the scavenging activity of the examined essential oils, extracts and pure predominant compounds on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

The DPPH assay was performed as previously described in the literature (Gordon, Paiva-Martins, & Almeida, 2001). Briefly, 5 solutions of Trolox (concentrations ranging from 0.005 to 0.025 μM) were made to react with DPPH and used to establish a calibration curve. The disappearance of DPPH colour was read spectrophotometrically at 515 nm using a Perkin–Elmer Lambda 2 spectrophotometer. The samples (accurately weighted) were mixed with 1 mL of 90 μM DPPH solution and the volume was adjusted to 4 mL with MeOH p.a. For each sample, the absorbance of four replicates was recorded. The RSC is expressed as μmol eq. Trolox/100 g of extract using the regression equation, obtained from the calibration curve:

$$y = -10.63x + 0.4701 \quad (R^2 = 0.9978)$$

2.6. Microorganisms

All strains were obtained from the Culture Collection of Industrial Microorganisms (CCMI) Laboratório de Microbiologia Industrial, Lisbon, Portugal.

Bacteria: *Escherichia coli* CCMI 270, *Listeria monocytogenes* CCMI 1106 *Micrococcus luteus* CCMI 322, *Mycobacterium smegmatis* CCMI 690, *Pseudomonas aeruginosa* CCMI 331, *S. aureus* CCMI 335, *Streptococcus faecium* CCMI 338. **Yeasts:** *C. albicans* CCMI 209, *Debaryomyces klockeri* CCMI 157 (Synonym of *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij var. *hansenii*), *Rhodotorula rubra* CCMI 43. *Rhodotorula mucilaginosa* is the current name for the species *R. rubra* (Bennett, 1990) but in this work the name registered in the collection will be referred.

2.6.1. Incubation conditions

Brain Heart Infusion (Merck, Darmstadt, Germany) was used as culture medium for bacteria, whereas Malt Extract Agar (Merck, Darmstadt, Germany) was used for yeasts.

The temperature and incubation time were variable according to the microorganism requirements. *M. luteus* was incubated at 30 °C for 24 h. *C. albicans* was incubated 30 °C for 48 h whereas *Debaryomyces klockeri* and *R. rubra* were incubated for 72 h at 30 °C. The other microorganisms were incubated at 37 °C for 24 h.

2.6.2. Screenings for antimicrobial activities

The antimicrobial activity of the compounds was determined by the broth/agar dilution methods (Muroi & Kubo, 1996), accordingly to the CLSI (formerly NCCLS) protocols (NCCLS 1997, NCCLS 2003).

The following concentrations were tested: 200, 100, 50, 25, and 12.5 μg mL⁻¹. A suspension of each microorganism (10⁵ cell/mL) was obtained by measuring optical density at 620 nm using a Lambda 2 Perkin Elmer spectrophotometer. 1 mL portions of the culture media were placed in the test tubes to which the successive dilutions of the oils/extracts samples were added; 20 μL of the cell suspensions were added to those mixtures. After incubation for 24 h - 48 h for bacteria, at 37 °C the microbial growth was examined. The same procedure was performed for yeasts, except that *D. klockeri* and *R. rubra* were incubated for 72 h. The results are expressed in Minimal Inhibitory Concentration (MIC), the weakest concentration of the samples yielding no visible growth. The bactericidal/fungicidal activities were determined by a sub-cultivation of the samples into normal culture media at appropriate temperature and incubation times (Benites et al., 2009).

5-Fluorocytosine (Sigma, Steinheim, Germany) was used as a synthetic antimycotic for a positive control, whereas Rifampicin (Sigma, Steinheim, Germany) was used as antibiotic positive control. The MIC of each compound was determined at least twice. Essential oils and *n*-hexane extracts from collections 1 to 4 were used in this study. Sample 3b was used in this work, i.e., essential oil and *n*-hexane extract of flowers of the plants collected at location 3. For simplicity, they will be referred to as sample 3 throughout this work. The essential oils and *n*-hexane extracts were dissolved in dimethylsulphoxide (DMSO) (Merck, Darmstadt, Germany). The predominant compounds thymol, carvacrol, methyl-carvacrol, *p*-cymene and γ -terpinene were also tested in the conditions described above.

3. Results and discussion

3.1. Chemical composition of the essential oil

Wild plants were collected in June 2006, in several locations of Madeira Island in zones of protection of the Laurisilva forest, characterized by high rainfall and high relative humidity, between 500 and 1000 m of altitude. Some variations in plant maturation were found, even though a direct correlation with altitude, humidity or sun light exposure was not possible to establish. Plants collected at Calheta and Santo da Serra (sample 1 and 4) had a much lower proportion of flowers (actually, bracts and flowers) to leaves than the other collections.

The content of the essential oils, expressed in mg of essential oil/100 g dried plant, is presented on Table 1.

Table 2 shows the list of chemical components of the investigated essential oils and their relative amounts, expressed as percentages. Major components are highlighted on that table. The yield shows variations which reflect the proportion of flowers to leaves on the analysed samples. By separating one of the collections (3) into leaves and flowers (3a and 3b, respectively), we found that essential oil accumulates mainly in the flowers. The same tendency was found for 1-hexacosanol isolated from the *n*-hexane extracts (Table 1). This compound represents the main difference between essential oils and *n*-hexane extracts. After its removal by precipitation, the respective chromatograms are very much alike. The composition of two of those extracts (from samples 1 and 2) is also presented in Table 2.

Data for typical essential oil contents and composition of *O. vulgare* subsp. *virens* are not abundant, although recently several papers related to this subspecies were published. Ferreira et al. (1998) refer that *O. vulgare* subsp. *virens* essential oil is not rich in carvacrol. However, Salgueiro et al. (2003) studied a carvacrol rich *O. vulgare* subsp. *virens* and its activity on *C. albicans*.

It has been pointed out (Figueredo, Cabassu, Chalchat, & Pasquier, 2006) that there are significant differences in the yield and composition of essential oils from populations of *O. vulgare* subsp. *virens* arising from environment factors, the most important of those being altitude, relating this to lack of water and short growing periods. Studies on *O. vulgare* subsp. *virens* letswaart from Italy, France and Portugal revealed markedly different chemical

Table 1
Plant contents in essential oil and *n*-hexacosanol.

Collection	mg essential oil/100 g dried plant	mg hexacosanol/100 g dried plant
1	890	111.18
2	1730	252.72
3	2410	251.07
4	700	48.65

Table 2

Chemical composition of essential oils (EO), expressed as percentages, obtained by hydrodistillation of aerial parts of *Origanum vulgare* subsp. *virens* from Calheta (1), Encumeada (2), Ponta do Sol (3) and Santo da Serra (4). The chemical composition of hexane extracts (Hex) is presented for samples 1 and 2.

Compounds	R1a	R1p	1		2		3		4	
			EO	Hex	EO	Hex	EO	EO		
<i>α</i> -Thujene	924	1029	1.3	1.08	0.34	0.47	0.4	0.63		
<i>α</i> -Pinene	930	1030	0.42	0.63	0.22	0.24	0.19	0.32		
1-Octen-3-ol	955	1147	1.1	0.47	0.39	0.53	0.3	1.21		
Sabinene	958	1123	0.2	v		0.17	v	0.2		
β -Pinene	963	1124	0.2	v		v	v	v		
Myrcene	975	1157	1.44	1.72	0.8	0.85	0.75	1.05		
<i>α</i> -Terpinene	1002	1187	2.39	2.50	1.46	1.36	1.43	1.7		
<i>p</i> -Cymene	1003	1276	6.93	12.49	5.91	5.72	4.61	11.33		
1,8-Cineole	1005	1206	0.35	0.48	0.28	0.25				
Cis- β -ocimene	1017	1236	5.34	6.10	0.58	1.14	0.56	2.51		
Trans- β -ocimene	1027	1236	0.65	0.22	v	0.18	0.52	0.32		
γ -Terpinene	1035	1250	20.49	20.79	8.66	10.31	10.38	19.61		
Trans sabinene hydrate	1037	1540	0.28	0.25					v	
Terpinolene	1064	1290	0.37	v					v	
Linalool	1074	1544	0.83	0.46	0.22	0.27	0.23	0.38		
Borneol	1134	1696	0.19		0.18	v	v	0.37		
Terpinen-4-ol	1148	1598	3.97		1.05	1.07	0.78	1.61		
<i>α</i> -Terpineol	1159	1692	0.21					v		
<i>Thymol methyl ether</i>	1208	1583	6.91	4.19	1.84	1.38	1.96	7.11		
<i>Carvacrol methyl ether</i>	1224	1583	4.36	2.85	4.13	2.91	2.89	1.19		
<i>Thymol</i>	1275	2180	19.37	13.38	55.0	50.3	58.0	30.96		
<i>Carvacrol</i>	1286	2212	1.39	0.45	6.75	2.86	1.14	1.36		
β -Caryophyllene	1414	1600	9.07	9.67	5.3	7.08	6.08	6.44		
<i>α</i> -Humulene	1447	1660	1.26	2.77	0.8	1.06	0.91	0.9		
γ -Murolene	1469	1684			0.54		0.25			
Germacrene D	1474	1684	3.54	6.40	0.34		2.48	3.91		
γ -Cadinene	1500	1751	1.77	3.21	0.55	0.63	0.79	1.78		
Calamenene	1503		0.17	0.52			v	0.29		
Bisabolene	1505		3.54	4.39	3.13	5.05	4.96	2.01		
δ -Cadinene	1509	1751	0.29	1.27	0.67	0.33	0.39	0.24		
Spathulenol	1551	2061	0.26					0.33		
Caryophyllene oxide	1561	1970	0.33	0.59				0.34		
Total			98.92	97.1	99.18	98.98	100	98.1		
<i>Phenolic Compounds</i>			<i>32.03</i>	<i>20.28</i>	<i>67.72</i>	<i>57.45</i>	<i>63.99</i>	<i>40.62</i>		

R1a and R1p: retention index relative to C₈–C₂₀ *n*-alkanes in OV101 (apolar) and Supelcowax 10 (polar) column, respectively. Compounds are listed in order of elution in OV101 column. v: vestige, blank: not detected. Phenolic compounds percentages are highlighted in italic.

compositions. The main constituents of the Italian oil were linalool (10.1–70.1%), β -caryophyllene (2.9–18.8%), carvacrol (<0.1–13.7%), terpinen-4-ol (<0.1–15.5%) and *α* -terpineol (<0.1–68.3%) (Melegari et al., 1995); and of the French oil, thymol (3.7–9.6%), β -caryophyllene (7.2–10.6%), sabinene (6.5–11.8%) and germacrene D (22.1–25.7%). Oil from Portugal differed from the others by the presence of greater amounts of the non-functionalized hydrocarbons δ -elemene (12.9%), β -caryophyllene (11.1%), *α* -terpineol (9.2%), germacrene B (6.6%), (*E*)- β -ocimene (6.6%) and (*Z*)- β -ocimene (3.7%). Ferreira et al. (1998) reported differences in essential oil composition depending on light and temperatures. Most of these studies refer to Mediterranean climate and its variations.

In Madeira Island, with a subtropical climate, some of these considerations do not apply – altitude does not imply lack of water, always abundant, or light although it means a sharp decrease in average temperature. In general, it can be stated that all tested samples from *O. vulgare* subsp. *virens* growing wild in Madeira belong to a thymol rich chemotype. The most remarkable feature of these essential oils is their high contents in thymol methyl ether (1.84 up to 7.11) and carvacrol methyl ether (1.19–4.36%). Indeed, all our oils were richer in carvacrol methyl ether than carvacrol itself. According to Figueredo et al. (2006) and references therein, nine *Origanum* species from the Mediterranean showed contents in these two ethers ranging from 0.1 to 0.5%, trace in most samples.

Only Hazzit, Baaliouamer, Faleiro, and Miguel (2006) refer to plants with noticeable amounts of these ethers: an *Origanum floribundum* with 6.9% of carvacrol methyl ether and a *Thymus guyonii* presenting 10.7% of thymol methyl ether. High levels of thymol methyl ether (16.3%), and carvacrol methyl ether (11.4%) were found in *Origanum vulgare* ssp. *gladulosum* from Algeria (Houmani, Azzoudj, Naxakis, & Skoula, 2002). *Satureja subspicata* growing wild in Croatia presents 8.83% of thymol methyl ether and shows good antimicrobial activity despite the low levels of free phenols (Skocibusic, Bezic, & Dunkic, 2006).

3.2. Antioxidant activity

In the DPPH assay, the ability of the investigated essential oils and extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its reduced form DPPH was investigated. All of the assessed samples were able to reduce the stable, purple-coloured radical DPPH into yellow-coloured DPPH. Despite variations in composition, the RSC of the essential oils was not much different from each other, ranging from 249.35 to 263.64 $\mu\text{mol eq. Trolox}/100\text{ g oil}$, and did not reflect the amount of phenolic components, to which the RSC is usually attributed (Table 3).

No direct correlation with each of free phenols, methylphenols or total phenolic compounds is possible even though the activity RSC of thymol is more than 10 fold the RSC of carvacrol, when the same method is applied to the pure compounds. The same trend was found by Yanishlieva et al. (1999) who used a different method, the autoxidation of purified triacylglycerols, to assess the antioxidant capacity of the two phenols. Thymol was found to be a much better antioxidant than carvacrol and the fact was attributed to the greater steric hindrance of the hydroxyl group of thymol. Kulisic et al. (2004) found similar RSC for both phenols in the DPPH method. In our study, the essential oils were less active than the extracts in *n*-hexane, but were more active than extracts in chloroform, ethyl acetate or even the rosmarinic acid rich methanol extract. The activities of the essential oils are around 250 $\mu\text{mol eq. Trolox}/100\text{ g oil}$, while the values found for RSC of solvent extracts were 150 ± 5 for chloroform extracts, 120 ± 3 for ethyl acetate and 110 ± 3 $\mu\text{mol eq. Trolox}/100\text{ g}$ for methanol.

3.3. Antimicrobial activity

The essential oils and *n*-hexane extracts of the four origanum collections were analysed for antimicrobial activity against 4 Gram-positive bacteria, 2 Gram-negative bacteria, 1 fast-acid bacterium and 3 yeasts. The antimicrobial activity of the compounds thymol, carvacrol, methyl-carvacrol, *p*-cymene and γ -terpinene present in the constitution of the essential oils was also evaluated. All essential oils, *n*-hexane extracts and the isolated compounds showed moderately activity when compared to the standard antibiotics (Tables 4 and 5). The essential oils did not differ remarkably in their activity against the tested microorganisms.

Table 3

Radical Scavenging Capacity of essential oils and *n*-hexane extract determined by the DPPH reaction.

Samples	RSC ($\mu\text{mol eq. Trolox}/100\text{ g EO}$)	RSC ($\mu\text{mol eq. Trolox}/100\text{ g extract}$)
1	249.35 \pm 1.24	341.90 \pm 2.66
2	262.99 \pm 2.33	327.84 \pm 1.34
3	263.64 \pm 1.76	–
4	259.31 \pm 1.12	–

There are some differences between the results obtained with the *n*-hexane extracts and the total essential oils, in several cases the extracts being unexpectedly more active than the essential oils.

For instance, the MICs of 25 $\mu\text{g mL}^{-1}$ obtained for *n*-hexane extracts of samples 1 and 2 against *M. smegmatis* is much lower than that obtained for the respective essential oils (200 and 100 $\mu\text{g mL}^{-1}$). Thymol showed the same activity, which suggests a correlation between this compound contents and *M. smegmatis* susceptibility. Pure methyl-carvacrol was also a very active compound.

The activity of all samples against the human pathogen, *S. aureus* seems to be related to the presence of thymol and carvacrol, both bactericidal at 100 $\mu\text{g mL}^{-1}$. The other pure compounds were inactive. The MICs obtained with the *n*-hexane extracts are lower than those obtained with the isolated compounds, which suggests a synergistic effect.

The human pathogen *E. coli*, was inhibited by essential oils 2 and 3 and, by *n*-hexane extracts 1, 2, 3 and 4. Thymol and carvacrol showed bactericidal activity at 100 $\mu\text{g mL}^{-1}$ and γ -terpinene was active at the higher concentration.

Thymol inhibited the growth of *P. aeruginosa* with cidal activity at 200 $\mu\text{g mL}^{-1}$, but no inhibition was observed with the essential oils or the *n*-hexane extracts. In the present study, carvacrol was not active against *P. aeruginosa*. The results obtained with thymol may be considered very interesting, since that microorganism is very resistant even to rifampicin (MIC = 200 $\mu\text{g mL}^{-1}$).

Recently, Cox and Markham (2007) studied the susceptibility and intrinsic tolerance of *P. aeruginosa* to carvacrol, terpinen-4-ol, α -terpineol, citral, geraniol, linalool, trans-cinnamaldehyde and eugenol (but not thymol) finding that only carvacrol caused damage to the outer membrane of *P. aeruginosa*. The intrinsic tolerance of *P. aeruginosa* strains to those plant volatile compounds was associated with an active efflux mechanism and the barrier function of the outer membrane. A proton transfer mechanism based on the presence of its phenolic hydroxyl group has been proposed to explain the depolarizing actions of carvacrol (Ben Afra, Combes, Preziosi-Belloy, Gontard, & Chalier, 2006; Utree, Bennink, & Moezelaar, 2002).

S. faecium showed no susceptibility to γ -terpinene and the other tested pure compounds were only bacteriostatic at the maximum tested concentration, 200 $\mu\text{g mL}^{-1}$. However, we observed bacteriostatic activity of the *n*-hexane extracts at 100 $\mu\text{g mL}^{-1}$, similar for all samples. For the essential oils, it was observed that samples 1 and 4, richer in phenols, have bactericidal activity, whilst samples 2 and 3, richer in hydrocarbons, have bacteriostatic activity at 100 and 50 $\mu\text{g mL}^{-1}$. Synergism between essential oil components will be further explored.

The antimicrobial activity against the food spoilage Gram-positive bacterium *M. luteus*, was detected for all essential oils and *n*-hexane extracts. Essential oil of samples 2 and 3 have bactericidal activity whereas, 4, the one richer in *p*-cymene, presents bacteriostatic activity, thymol and carvacrol showed bactericidal activity. Although *M. luteus* is non-pathogenic and usually regarded as a contaminant, it should be considered as an emerging nosocomial pathogen in immunocompromised patients. *M. luteus* is resistant to reduced water potential and can tolerate drying and high salt concentrations.

C. albicans is a harmless commensal yeast-like fungus in healthy humans which can cause systemic infections under immunocompromised individuals (Manohar et al., 2001). Thus, our interest in evaluate the potential of essential oils against this opportunistic microorganism. *C. albicans* was inhibited with fungicidal activity by *n*-hexane extracts of samples 3 and 4 at 200 $\mu\text{g mL}^{-1}$. Extracts of samples 1 and 2, and their essential oils were fungistatic at 200 $\mu\text{g mL}^{-1}$. Carvacrol was found fungistatic at 50 $\mu\text{g mL}^{-1}$. All other

Table 4
Antimicrobial activity of Origanum essential oils and extracts.

Microorganisms	Antimicrobial activity of Origanum essential oils and <i>n</i> -hexane extracts ($\mu\text{g mL}^{-1}$)							
	1		2		3		4	
	EO	<i>n</i> -Hexane	EO	<i>n</i> -Hexane	EO	<i>n</i> -Hexane	EO	<i>n</i> -Hexane
<i>C. albicans</i>	>200	200 ^a	200 ^a	100 ^a	200 ^a	200	>200	200
<i>D. kloeckeri</i>	200	100 ^a	100	50 ^a	100 ^a	100 ^a	100	100 ^a
<i>E. coli</i>	>200	200	200	200	200	200	>200	200
<i>L. monocytogenes</i>	100 ^a	200 ^a	100 ^a	100 ^a	100 ^a	200 ^a	100 ^a	200 ^a
<i>M. luteus</i>	200 ^a	200	100	100	100	200	100 ^a	200
<i>M. smegmatis</i>	200	25 ^a	100	25 ^a	100	50 ^a	100 ^a	100
<i>P. aeruginosa</i>	>200	>200	>200	>200	>200	>200	>200	>200
<i>R. rubra</i>	200	100 ^a	200	100 ^a	200	100 ^a	200	100 ^a
<i>S. aureus</i>	100	50 ^a	100	50	100	50	100	50
<i>S. faecium</i>	100	100 ^a	100 ^a	100 ^a	50 ^a	100 ^a	50	100 ^a

^a Bacteriostatic/fungistatic.

pure components were fungistatic at $100 \mu\text{g mL}^{-1}$, with the exception of γ -terpinene, which was inactive. These results agree with those obtained by Manohar et al. (2001) with an origanum essential oil which inhibited the growth of *C. albicans*. The same authors attributed the antifungal activity to carvacrol. Tampieri et al. (2005) studied the inhibition of *C. albicans* by selected essential oils and their major components, concluding that, among phenols, carvacrol is the most effective inhibitor. The antimicrobial effect of the essential oils towards *L. monocytogenes* is stronger than those obtained with *n*-hexane extracts, suggesting a synergistic effect. Among the pure compounds, thymol and carvacrol showed antibacterial activity. According to Yamazaki, Yamamoto, Kawai, and Inoue (2004) those compounds were found to have strong antilisterial properties. Periago, Delgado, Fernández, and Palop (2004) verified that the combination of carvacrol and cymene resulted in an increased antibacterial effect on the growth and a synergistic effect on the viability of *L. monocytogenes* compared with the natural compounds applied separately. The results obtained with this microorganism are very relevant, due to its ability to grow at refrigeration temperatures, over a wide range of pH values (4.4–9.6) and in the presence of high salt content (Hazzit et al., 2006) surviving mild preservation treatments, features that make it difficult to eliminate this microorganism from foods.

Thymol has a MIC value of $50 \mu\text{g mL}^{-1}$ towards *D. kloeckeri*, the lowest value when compared to those obtained with the other isolated compounds, essential oils and the *n*-hexane extracts, excepting for sample 2 which was also fungistatic at that concentration. *D. kloeckeri* is presently considered synonymous of *D. hanseni*, the most common species of yeast found in all types of cheeses, dairies and in brine, exhibiting high tolerance to salt and organic acids at low temperature. It may cause undesirable sensory

changes in yoghurts, ice creams, fish, shellfish, due to the formation of off-odours and off-flavours.

We detected antimicrobial activity of all origanum essential oils against *R. rubra*, a common airborne contaminant of skin, lungs, urine and faeces. The essential oils were less active than the respective hexane extracts, which were all bacteriostatic and showed the same activity of thymol and carvacrol. Methyl-carvacrol and *p*-cymene showed bacteriostatic effect, whereas γ -terpinene was inactive. Alma et al. (2003) did not observe antifungal activity of *Origanum syriacum* L. essential oil against that microorganism; however, their oil had precisely γ -terpinene as the most abundant component, with carvacrol in second place and very little thymol. The interest in the results obtained with *R. rubra*, a carotenoid-synthesizing, acid-tolerant, aerobic yeast, is its ability to use fruits and other nutrient rich foodstuffs and cause spoilage by producing off-flavours and odours and colour changes. Colonies present the aspect of pink or red slimy globules, thus degrading the economic value of beverages and yogurts.

Burt (2004) revised the antimicrobial activity of essential oils and modes of antibacterial action of essential oils, which include impairment of a variety of enzyme systems including those involved in energy production and structural component synthesis. Phenolic compounds are known to cause structural and functional damage to plasma membranes since the permeability of cell membranes depends on the hydrophobicity of the solutes that have to cross the membrane and on the composition of the membrane. Quantitative variations in the activity of essential oil are thus expected against different bacteria, especially when bacteria with different Gram are considered. Unlike many antibiotics, the hydrophobic constituents of origanum essential oils are capable of gaining access to the periplasm of Gram-negative bacteria through

Table 5
Antimicrobial activity of pure major components of Origanum and standard antibiotics.

Microorganisms	Antimicrobial activity of pure major components of Origanum and standard antibiotics ($\mu\text{g mL}^{-1}$)						
	Thymol	Carvacrol	Methyl-carvacrol	<i>p</i> -cymene	γ -Terpinene	Rifampicin	5-Fluorocytosine
<i>C. albicans</i>	100 ^a	50 ^a	100 ^a	100 ^a	>200	(n.t.)	5.0
<i>D. kloeckeri</i>	50 ^a	100 ^a	100 ^a	100 ^a	200 ^a	(n.t.)	5.0
<i>E. coli</i>	100	100	>200	>200	200	1.0	(n.t.)
<i>L. monocytogenes</i>	100	200	>200	>200	>200	0.07	(n.t.)
<i>M. luteus</i>	100	200	>200	100 ^a	>200	0.01	(n.t.)
<i>M. smegmatis</i>	25 ^a	200 ^a	50 ^a	100 ^a	>200	0.5 ^a	(n.t.)
<i>P. aeruginosa</i>	200	>200	>200	>200	>200	200 ^a	(n.t.)
<i>R. rubra</i>	100 ^a	100 ^a	200 ^a	200 ^a	>200	(n.t.)	5.0 ^a
<i>S. aureus</i>	100	100	>200	>200	>200	0.001	(n.t.)
<i>S. faecium</i>	200 ^a	200 ^a	200 ^a	200 ^a	>200	0.5 ^a	(n.t.)

(n.t.): not tested.

^a Bacteriostatic/fungistatic.

the porin proteins of the outer membrane (Helander et al., 1998). Thymol and carvacrol were also found to disintegrate the outer membrane of *E. coli* and *Salmonella typhimurium* at levels close to the MIC (Helander et al., 1998).

The structural requirements for the antimicrobial activity of carvacrol have been established (Veldhuizen, Tjeerdma-van Bokhoven, Zweijter, Burt, & Haagsman, 2006), the presence of an aromatic hydroxyl group being essential for the activity, together with moderate hydrophobicity. The same considerations can be applied to thymol: both have been previously described as able to disrupt the bacterial membrane, by affecting both the pH gradient and the electron flow across the membrane (Helander et al., 1998; Lambert, Skandamis, Coote, & Nychas, 2001). This was shown to damage the cells irreversibly. The biological precursor of carvacrol, *p*-cymene, is not an effective antibacterial agent when used alone; however, when combined with carvacrol, synergism has been observed (Ultee et al., 2002). The greater efficiency of *p*-cymene incorporation into the lipid bilayer very likely facilitates the transport of carvacrol across the cytoplasmic membrane.

In the present study, the main difference in composition between essential oils and hexane extracts is the presence of significant amounts of *n*-hexacosanol. This pure compound was tested and it was found totally inactive over the ten assayed microorganisms. The long chain primary alcohols C₆ to C₂₀ antimicrobial activity was studied by Kubo, Muroi, and Kubo (1995) concluding that for chain lengths over C₁₆, no activity was found over all the tested microorganisms with a sudden drop in activity from C₁₆ to C₁₇. However, previous researchers (Moosbrugger, Bischoff, Beck, Luu, & Borg, 1992) reported *n*-hexacosanol and similar long chain alcohols to be able to interfere with the cell membrane properties, so an effect of this compound similar to that proven to inactive *p*-cymene is not to be discarded.

We have demonstrated that *Origanum* essential oils, their *n*-hexane extract and the predominant isolated compounds inhibit the growth of several spoilage food and human pathogenic microorganisms.

The oils studied in the present work show similar activity, even though their content in carvacrol is very small, thymol being the major phenolic in all scrutinized samples. Recent studies (Sari et al., 2006) on *Origanum glandulosum* from Algeria report identical findings with a fairly similar susceptibility of gram-negative and gram-positive bacteria and yeast to all tested essential oil regardless of the predominance of thymol or carvacrol, and a very poor susceptibility of *P. aeruginosa*.

4. Conclusions

Wild *O. vulgare* subsp. *virens* from Madeira Island belong to a thymol rich chemotype.

The essential oils and *n*-hexane extracts have greater radical scavenging capacity than the more polar extracts, probably due to the high contents in thymol, which demonstrated the highest activity in the DPPH assay. We hope to be able to follow this trend in the antimicrobial assays, even though the bioactivity of thymol and carvacrol over a large range of microorganisms was not very different.

All *Origanum* samples under study show a large content in non-esterified *n*-hexacosanol, mainly accumulated in bracts and flowers, which although not showing by itself any activity as antimicrobial or antioxidant deserves to have its role in cell membrane disruption further explored.

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