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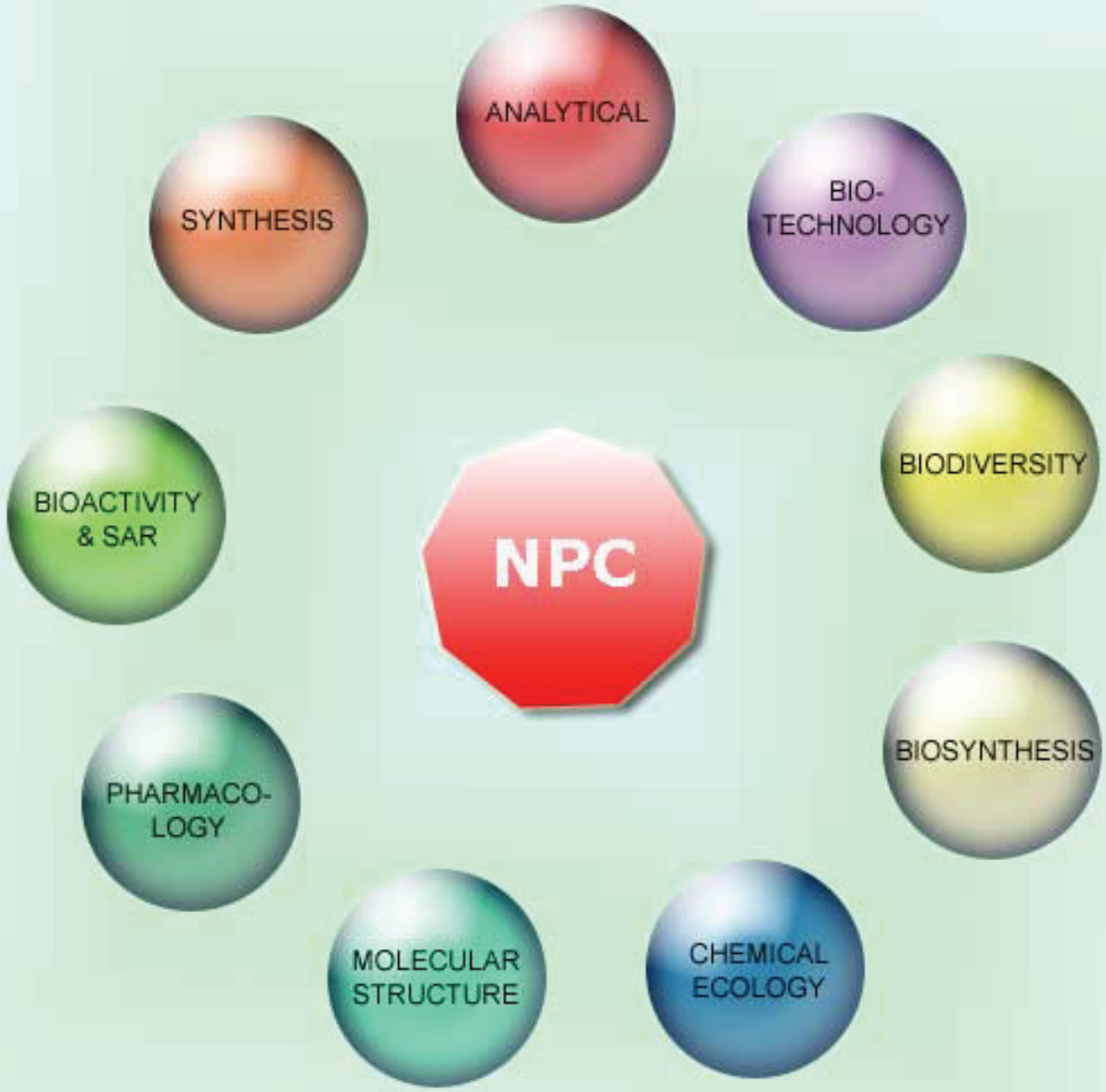

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Exploring the Effect of the Composition of Three Different Oregano Essential Oils on the Growth of Multidrug-Resistant Cystic Fibrosis *Pseudomonas aeruginosa* Strains

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Oregano essential oils (EOs) could represent interesting therapeutic strategies to treat multidrug-resistant (MDR) pathogens as *Pseudomonas aeruginosa*, responsible for respiratory infections in cystic fibrosis (CF) patients. There could be a great variability in EOs composition when extracted from different plant species. The aim of this study was to chemically characterize and to test EOs, extracted from *Origanum compactum*, *Origanum vulgare* and *Origanum vulgare var. hirtum*, for *in vitro* antimicrobial activity against a panel of twenty MDR *P. aeruginosa* strains isolated from CF patients. EOs main components were carvacrol (71.8-73.8-47.1%), thymol (1.6-2.3-21.5%), *p*-cymene (11.6-7.4-10.8%) and γ-terpinene (1.7-3.1-8.4%). In general, the EOs showed inhibitory activity even at low concentration: 0.5% (v/v) OvEO and OhEO were able to inhibit the 80% of *P. aeruginosa* strains. Furthermore, the three EOs killed at least 75% of the strains at concentrations lower than 1% (v/v). Average MIC and MBC values were not significantly different. Similar levels of OEOs antimicrobial activities might be related to the fact that the main chemical class (i.e. carvacrol/thymol) is represented in quite similar percentages. Hence, the results of the present study shed light on a carvacrol/thymol-rich EO with a well-represented monoterpene hydrocarbons class as promising standardized antimicrobial herbal product.

Keywords: Essential oils, *Origanum vulgare*, *Origanum compactum*, *Pseudomonas aeruginosa*, Multidrug resistance, Cystic fibrosis.

The widespread emergence of multidrug resistant (MDR) microorganisms has renewed the interest for alternative compounds with multiple target sites, potentially able to limit the risk of developing antibiotic resistance (Ab-R). Indeed, it is more difficult for bacteria to develop resistance to a multicomponent drug than to single chemical entities directed toward a given molecular target as often are the antibiotics [1]. In general, the essential oils (EOs) are mixtures of interacting compounds belonging to different classes such as phenols, alcohols, aldehydes, ketones or hydrocarbons [2] that could attack several bacterial molecular targets. EOs are products of plant metabolism and their formulation is reported to be influenced by geographical plant position (*e.g.* climatic conditions and soil composition), environmental parameters, phenological stages and extraction techniques [3, 4]. Thus, a great variability in the composition of EOs especially belonging to different species might exist. From this viewpoint, one of the most promising medicinal plant is *Origanum* L., (Lamiaceae), a genus embedding at least 38 species of annual and perennial herbs, which is mainly restricted to the eastern part of the Mediterranean area, Europe, North Africa and Asia [4]. *Origanum vulgare* L. shows a great morphological and intraspecific variability with its six subspecies, including the *vulgare* L. (Ov) and the *hirtum* (Oh) [5] that are the object of this study. *Origanum compactum* (Oc) grows in North Morocco where it is traditionally used in culinary and medical preparations [6]. Oregano EOs contain both carvacrol and thymol and a variable quantities of *p*-cymene and γ-terpinene that have been shown to inhibit *Escherichia coli*, and serovars *enteritidis*, *choleraesuis*, and *typhimurium* of *Salmonella enterica* [7]. Indeed, many bacteria, especially pathogens, exhibit high sensitivity to Oregano EOs [8-10]. Among bacterial pathogens, *Pseudomonas aeruginosa* is responsible for respiratory infections in cystic fibrosis (CF) patients showing an increasing level of multidrug resistance [11] also due to biofilm formation [12-13]; hence, it represents a good model to test new antimicrobial compounds. Thus, the aim of this study was to compare the *in vitro* antimicrobial efficacy of three different oregano oils against a panel of human bacterial pathogens belonging to the species *P. aeruginosa*, consisting of clinical multidrug resistant strains isolated from cystic fibrosis patients. In fact it appears pivotal to be able to suggest a standardized composition of oregano EO to allow a formulation of an antimicrobial phytomedicine.

The molecular characterization of each *P. aeruginosa* strain revealed that the twenty bacterial isolates corresponded to different strains (data not shown). The Ab-R profile of each *P. aeruginosa* strain was determined using a panel of thirteen different antibiotics, belonging to four different classes and listed in supplementary Table 1 and summarized in Figure 1.

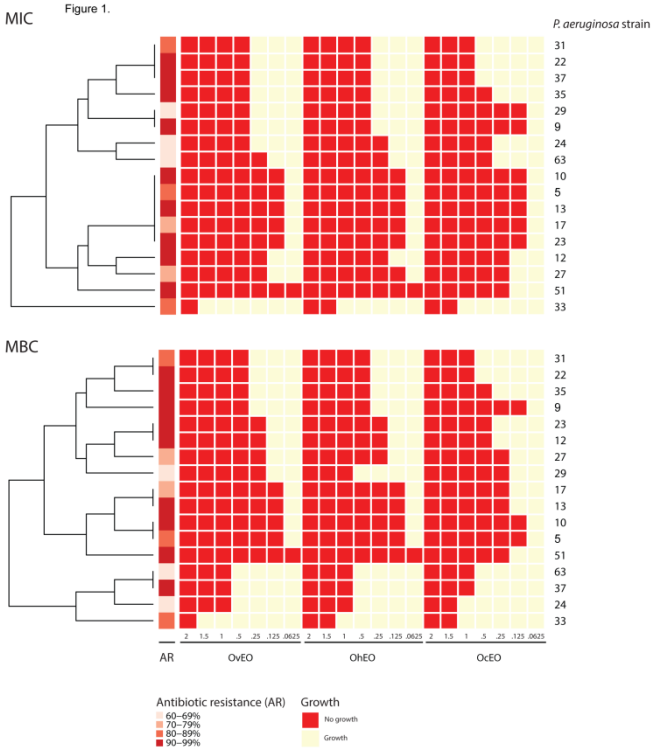

Figure 1: MIC and MBC heatmaps
Upper) Heatmap showing the three Oregano EOs concentrations inhibiting the growth of seventeen *P. aeruginosa* strains and the percentage of tested antibiotics to which each strain is resistant.
Lower) Heatmap showing the three Oregano EOs concentrations killing seventeen *P. aeruginosa* strains and the percentage of tested antibiotics to which each strain is resistant.

In general, most strains were resistant to multiple antibiotics (belonging to at least two major classes of antibiotics); hence, according to the definition of the American Cystic Fibrosis Foundation [14], they can be defined as MDR. The percentage of antibiotics to which each strain was resistant (R) ranged from 61.5% to 92.3%. In particular, *P. aeruginosa* strains revealed a high degree of resistance (95-100%) against aminoglycosides, fluoroquinoles and cephalosporins, IMI and TCC. Different degrees of resistance (65-85%) for other β-lactams antibiotics were recorded whilst all 20 *P. aeruginosa* strains were sensitive (S) to colistin.

The OvEO composition has been already reported [9], whereas the composition of the other two oregano oils was determined as described in Experimental. Constituents and their principal classes of the three oregano oils are reported in supplementary Table 2, whose analysis revealed that the composition of the three EOs was quite different and a total of 39 compounds was detected. In the case of *O. vulgare* L. *var. hirtum,* total identified constituents were 99.8% and major constituents were represented by oxygenated monoterpenes (79.4%) being carvacrol the main volatile (73.8%). Monoterpene hydrocarbons were 15.9%, mainly *p*-cymene (7.4%). Sesquiterpene hydrocarbons were 3.8% and oxygenated sesquiterpenes were 0.6%. Total identified constituents of *O. compactum* L. were 99.8%. These volatiles were characterized by 72.9% of oxygenated monoterpenes, mainly carvacrol (47.1%) and thymol (21.5%) and monoterpene hydrocarbons 24.2%, being 10.8% *p*-cymene. The 2.1% of the volatiles were sesquiterpene hydrocarbons and oxygenated sesquiterpenes were 0.4%. Data from supplementary Table 2 were submitted to principal component analysis (PCA), and the results are shown in supplementary Figure 1. The vectors accounting for OvEO and OhEO are differentially oriented than that of OcEO. In particular, both EOs from Spain (OvEO and OhEO) might be categorized as carvacrol-rich chemotype, as they exhibited high amounts of carvacrol (71.80 and 73.80%) and low percentages of thymol (1.60 and 2.30%), whilst OcEO from Morocco is a chemotype with a prevalence of carvacrol/thymol (47.10 and 21.50%). Interestingly, PCAs carried out excluded the two main constituents (carvacrol and thymol) and also *p*-cymene, still grouped OvEO and OhEO together, separated from OcEO, mainly in relation to β-caryophyllene and ɣ-terpinene (supplementary Figure 2). In any case, the main constituent class (Oxygenated monoterpenes) is quite similarly represented in the three EOS (77.2% for OvEO, 79.4% for OhEO and 72.8% for OcEO) whilst the monoterpene hydrocarbons (*p*-cymene, ɣ-terpinene) class was slightly different (19.2% for OvEO, 15.9% for OhEO and 24.2% for OcEO).

The antimicrobial activity of different concentrations of the three EOs was tested for each of the 20 *P. aeruginosa* strains listed in supplementary Table 1 by determining both MIC and MBC. Data obtained at 48 h of incubation in presence of each EO are shown in supplementary Table 3 and summarized in supplementary Table 4 and Figure 1. Results obtained at 24 h and 72 h were consistent with those at 48 h (data not shown). The analysis of data reported in Table S3 and S4, and in Figure 1 revealed that the MIC and MBC values of each EOs resulted very similar between them.

Moreover, the three oregano EOs exhibited antibacterial activity against all strains at a similar extent with no statistical significance (data not shown). Three strains (4, 7 and 8) were resistant to the highest tested concentration; then the MICs and MBCs values could not be determined and these strains were excluded from the further analyses. However, interestingly, these strains were intermediate resistant (I) to aztreonam (strain 4 was also sensitive to ampicillin) suggesting that the synergistic effects (EOs plus antibiotics) should be investigated. In fact some combinations could boost the bactericidal effect with subsequent lowering the needed concentrations and the side effects risk [15].

In general, the EOs showed their inhibitory activity even at low concentration (Figure1); after 48 h, 0.5% (v/v) OvEO and OhEO were able to inhibit the 80% of all *P. aeruginosa* strains. Furthermore, OvEO and OhEO concentrations lower than 1% (v/v) were also sufficient to kill the 80% of the strains. The OcEO was less active inhibiting the 65% and killing the 75% of the strains at concentrations lower than 0.5% (v/v) and 1%, respectively. Differences between the average values for MICs and MBCs were not statistically significant (data not shown) even if a slightly minor antimicrobial activity resulted related to the OcEO (supplementary Table 3). For four strains (n = 22, 31, 37, 63) the MICs resulted two-fold lower for OvEO and OhEO respect to OcEO up to a MIC of 0.06% against 0.25% for one strain (n = 51). Minor average MICs (0.41 and 0.36 % v/v) belonged to the OvEO and OhEO, which are characterized by a carvacrol-rich composition (71.8 and 73.8%, respectively). Both of them were also effective up to a 0.06 % v/v concentration whilst the OcEO (carvacrol 47.1%) had an average MIC of 0.45 % v/v and it was active up to a concentration of 0.12 % v/v. However, the presence of a high thymol concentration in the OcEO (21.5% against 1.6 and 2.3% for OvEO and OhEO respectively) might have contributed to the similar MIC values for the three EOs. In fact, structure of the thymol is very similar to the carvacrol one and the different position of the hydroxyl group on the phenolic ring seemed not to be related to the antibacterial activity level [16]. Thymol and carvacrol were reported exerting similar antibacterial activity against *P. aeruginosa* and both of them carried out their activity by disrupting cell membrane [15].

Table 1: Major components and MICs of Oregano EOs tested *in vitro* against *P. aeruginosa* strains.

Origanum spp	**Main component** (%)	**MIC** (%, v/v) [a]	**References**
O. vulgare L.	Carvacrol (67.1); *p*-cymene / γ-terpinene (13.8 / 7.7)	0.06	[18]
	not reported	0.16	[19]
	Carvacrol / Thymol (71.8 / 1.6); *p*-cymene / γ-terpinene (11.6 / 1.7)	0.41 [b]	This study
O. vulgare L. *var. hirtum*	Thymol/Carvacrol (61.9 / 15.0); γ-terpinene / *p*-cymene (6.4 / 4.4)	> 0.1 [c]	[20] [d]
	Thymol/Carvacrol (49.0 / 14.5); γ-terpinene / *p*-cymene (13.4 / 4.7)		
	Thymol/Carvacrol (49.2 / 11.1); γ-terpinene / *p*-cymene (14.1 / 6.3)		
	Thymol/Carvacrol (52.9 / 1.2); γ-terpinene / *p*-cymene (12.0 / 12.0)		
	Thymol/Carvacrol (57.1 / 6.8); γ-terpinene / *p*-cymene (18.6 / 4.4)		
	Thymol/Carvacrol (24.5 / 0.1); γ-terpinene / *p*-cymene (34.8 / 9.4)		
	Carvacrol / Thymol (21.9 / 18.2); *p*-cymene / γ-terpinene (2.8 / 2.4)	> 0.01 [c]	[21]
	Thymol / α-terpineol (26.7 / 15.1); γ-terpinene / *p*-cymene (4.6 / 1.2)		
	Linalyl acetate / Linalool (15.9 / 12.5); γ-terpinene / *p*-cymene (4.9 / 2.0)		
	Carvacrol / Thymol (73.8 / 2.3); *p*-cymene / γ-terpinene (7.4 / 3.1)	0.36 [b]	This study
Origanum compactum L.	Carvacrol / Thymol (29.7 / 22.1); *p*-cymene / α, β and γ-terpinene (11.7 / 20.2)	2.19 [b]	[10] [d]
	Carvacrol / Thymol (37.8 / 19.8); γ-terpinene / *p*-cymene (17.0 / 11.3)	1.00	[6]
	Carvacrol / Thymol (47.1 / 21.5); *p*-cymene / γ-terpinene (10.8 / 8.4)	0.45 [b]	This study

[a] In the studies MICs have been reported in the units mg/mL, mg/L, % (v/v), µ l/L and µg/ml. For ease of comparison these have been converted to % (v/v), whereby it was assumed that EOs have the same density as water. [b] MIC mean value. [c] Maximum concentration tested that was not effective against any isolates. [d] agar dilution method assay.

Furthermore, *p*-cymene (OvEO 11.6%, OhEO 7.4% and OcEO 10.8%) and ɣ-terpinene (OvEO 1.7%, OhEO 3.1% and OcEO 8.4%) could increase the antimicrobial effect of the OcEO compelling the action of carvacrol and thymol. The four compounds are biologically associated; indeed, both *p*-cymene and ɣ -terpinene represent the precursors of carvacrol and thymol and synergism between carvacrol and *p*-cymene against *B. cereus* has been reported in an *in vitro* study [17].

The heterogeneity of the studies on the EOs antibacterial activity limited their comparison: a selection of oregano EOs MICs tested *in vitro* against *P. aeruginosa* was presented in Table 1 [6,1018-21].

In most cases the range of the MICs appeared quite narrow and comparable with the present study. Unfortunately, most studies did not perform the EOs chemical composition limiting the possibility to speculate on the relationship between antimicrobial activity and presence of carvacrol and other constituents. In general, lower MICs were reported for carvacrol-rich EOs (> 65%) rather than for the EOs with a carvacrol concentration less than the40%. Interestingly, one study on *O. compactum* [6] reported antimicrobial activity and chemical composition similar to the ones of the present study suggesting again a potential role of the monoterpene hydrocarbons (*p*-cymene and γ-terpinene).

The EOs antibacterial activity is not easily imputable to one or a few active principles, because they are constituted by many different chemical compounds [2] and also less-represented molecules might be significantly responsible for the EOs activity. The oregano EOs major (*i.e.* carvacrol and thymol) and minor (*e.g.* *p*-cymene and ɣ-terpinene) components might synergistically contribute to the respective antibacterial activities. Indeed, even though the three oregano EOs exhibit different chemical composition, their MIC and MBC are very similar; this might be related to the fact that the main chemical class (*i.e.* carvacrol/thymol) is represented in quite similar percentages in the three EOs. Hence, the results of the present study suggested that a carvacrol/thymol-rich oregano EO with a well-represented monoterpene hydrocarbon class could be a corner stone to standardize an herbal medicinal product with a broad spectrum of antimicrobial effects. A toxicological screening is fundamental before recommending a possible clinical use. In fact, the major challenges is to find compounds with sufficiently low MICs and toxicity, and high bioavailability for effective and safe use in humans and animals. The oregano EO tolerability could increase if properly channeled in specific pharmaceutical forms [22].

Experimental

Bacterial strains: The panel of 20 *P. aeruginosa* strains tested in this work (supplementary Table 1) was isolated from different patients; each strain was maintained at -80°C under glycerol (25% v/v) stock, and grown on Columbia blood agar (Thermo Scientific, Oxoid SpA, Strada Rivoltata, 20090 Rodano (MI) - Italy) at 37°C for 24 hours. Bacterial strains were identified using Matrix Assisted Laser Desorption Ionization Time-of-Flight (Maldi-Tof VITEK MS, bioMérieux Italia Spa, Italy). *P. aeruginosa* strains were typed through BOX-PCR fingerprinting.

Antibiotic resistance profiling: Susceptibility to clinically-relevant antibiotics selected across different antimicrobial families, was evaluated. In particular, the following antibiotics were tested: gentamicin (GEN) and tobramycin (TOB; Aminoglycosides), ampicillin (AMP), aztreonam (ATM), ceftazidime (CAZ), cefepime (CEF), levofloxacin (LVX), meropenem (MER), ticarcillin/clavulanic (TCC) and piperacillin/tazobactam (TZP; β-lactams), ciprofloxacin (CIP) and imipenem (IMI; Fluoroquinoles), and colistin (COL; Polymyxins). *In vitro* antibiotic susceptibility has been tested by disk diffusion. Results were interpreted according to the available EUCAST breakpoint tables [23].

Oregano essential oils: The *O. vulgare* L. EO was purchased from Prodotti Phitocosmetici Dott. Vannucci (di Vannucci Daniela e C. Sas, Prato, Italy) whilst the *O. compactum* L. and *O. vulgare* L. *var. hirtum* were purchased from Organic Trading Florisco (info@florisco.it). EOs were all extracted by steam distillation method from plants cultivated in Spain (OvEO and OhEO) or in Morocco (OcEO).

Determination of essential oil composition: Gas chromatographic (GC) analyses were accomplished with an HP-5890 series II instrument equipped with a HP-5 capillary column (30 µm × 0.25 mm, 0.25 µm film thickness), working with the following temperature program: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and detector temperatures, 250°C; carrier gas, nitrogen (2 mL/min); detector, dual flame ionization detection (FID); split ratio, 1 : 30; injection, 0.5 µL. The identification of the components was performed, for both columns, by comparison of their retention times with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of n-hydrocarbons and on computer matching against commercial and homemade library mass spectra built from pure substances and components of known samples and MS literature data [9]. Gas chromatography-electron impact mass spectrometry (GC-EIMS)

analyses were performed with a Varian CP 3800 gas chromatograph (Varian, Inc. Palo Alto, CA) equipped with a DB-5 capillary column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany; 30 m × 0.25 mm, coating thickness 0.25 mm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature at 250 and 240°C, respectively, oven temperature being programmed from 60 to 240°C at 3°C/min, carrier gas, and helium at 1 mL/min, split less injector. The molecular weights were confirmed by gas chromatography-chemical ionization mass spectrometry (GC-CIMS), using methanol as chemical ionization gas.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration: MIC and MBC were determined in TSB added with the EO in concentration two-fold diluted from 2.00% to 0.06% (v/v) and the same volume of dimethylsulphoxide (DMSO), sterilized by filtration through filters with a pore diameter of 0.22 µm (Sartorius Italy Srl, Monza e Brianza, Italy). Standard determination of MIC, in broth micro-dilutions, was performed. Microtiter plates containing EOs serial dilutions were inoculated with 100 µL of bacterial suspensions with approximately 2×10^6 CFU/mL in a final volume of 200 µL. Negative control contained 200 µL TSB and two positive controls TSB and DTSB (1% of DMSO) inoculated with 100 µL of the bacterial suspension, respectively. A further negative control was set up using an antibiotic able to inhibit the growth of the tested bacteria. Microplates were incubated at 37°C aerobically. The Infinite 200 PRO multimode reader (Tecan, Männedorf, Switzerland), was used to detect density (using OD600) at 24, 48 and 72 h. At time "0" and at 24 h intervals up to 72 h, from each tube, 10 µL of the suspension were spread on TSA plates and were incubated at 37°C aerobically; afterwards, the CFU was determined. All assays were performed in triplicate.

Statistical analyses: Average and standard deviations of MIC values were estimated and compared by using Analysis of Variance (ANOVA) with Fisher's least significance differences procedure at a significance level of 0.05. To evaluate whether the EOs constituents identified are useful in reflecting the chemical relationships between species, a principal component analysis (PCA) was performed using the PAST software.

Supplementary data: Antibiotic resistance profile, Oregano EOs composition, MIC and MBC of each strain are also available.

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