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Essential Oil from *Origanum vulgare* Completely Inhibits the Growth of Multidrug-Resistant Cystic Fibrosis Pathogens

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Essential oils (EOs) are known to inhibit the growth of a wide range of microorganisms. Particularly interesting is the possible use of EOs to treat multidrug-resistant cystic fibrosis (CF) pathogens. We tested the essential oil (EO) from *Origanum vulgare* for *in vitro* antimicrobial activity, against three of the major human opportunistic pathogens responsible for respiratory infections in CF patients; these are methicillin-resistant *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*. Antibiotic susceptibility of each strain was previously tested by the standard disk diffusion method. Most strains were resistant to multiple antibiotics and could be defined as multi-drug-resistant (MDR).The antibacterial activity of *O. vulgare* EO (OEO) against a panel of 59 bacterial strains was evaluated, with MIC and MBC determined at 24, 48 and 72 hours by a microdilution method. The OEO was effective against all tested strains, although to a different extent. The MBC and MIC of OEO for *S. aureus* strains were either lower or equal to 0.50%, v/v, for *A. xylosoxidans* strains were lower or equal to 1% and 0.50%, v/v, respectively; and for *S. maltophilia* strains were lower or equal to 0.25%, v/v. The results from this study suggest that OEO might exert a role as an antimicrobial in the treatment of CF infections.

Keywords: Essential Oils, *Origanum vulgare*, Multidrug resistance, Cystic Fibrosis, Methicillin-resistant *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*.

Leading causes of morbidity and mortality in cystic fibrosis (CF) are respiratory tract infections caused by human pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA), *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* [1]. In the United States, the prevalence of MRSA, *S. maltophilia*, and *A. xylosoxidans* in CF patients is in the order of 22.6%, 15.8% and 6%, respectively. Two studies in Italy have reported a prevalence of 16% of *A. xylosoxidans* infection [2] and of 7% for MRSA [3]. These bacteria often show an increasing level of multidrug resistance (MDR) hampering the treatment of CF-associated infections with conventional antimicrobial therapies [1]. Thus, new therapeutic strategies need to be discovered. Much attention is paid to bioactive compounds derived by plants that are known to possess antimicrobial properties and are used in traditional medicine to treat several diseases. In particular, essential oils (EOs) play an important role in the protection of plants and have been empirically used for centuries to treat upper respiratory tract infections such as pharyngitis, bronchitis and sinusitis [4]. Furthermore, in many cases the EO activity results from the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers and hydrocarbons found in EOs [5]. It is likely that it will be more difficult for bacteria to develop resistance to the multi-component EOs than to common antibiotics, which are often composed of only a single molecular entity [6], since several molecular targets would need to adapt to overcome the effects of the oil [7].

The activity of EOs against bacteria, fungi and viruses has been tested in many studies and indeed, many bacteria, especially pathogens, exhibit high sensitivity to EOs mainly extracted from thyme, oregano and cloves [8-10]. We have previously performed a preliminary analysis of EOs from six aromatic medicinal plants (*Eugenia caryophyllata*, *Origanum vulgare*, *Rosmarinus officinalis*, *Lavandula hybrida*, *Melaleuca alternifolia* and *Thymus vulgaris*) revealing that, despite their different chemical composition, all of them were able to inhibit the growth of representative strains of members of the *Burkholderia cepacia* complex (Bcc) [11].

The antibacterial activity of *Origanum vulgare* EO (OEO) has been attributed to the phenolic components, such as thymol and carvacrol [5], which are able to inhibit some pathogenic bacterial strains, including *Escherichia coli*, and serovars *enteritidis*, *choleraesuis*, and *typhimurium* of *Salmonella enterica* [12]. Previous studies have reported that OEO shows antimicrobial activity both *in vitro* and *in vivo* against *S. aureus* [13-16]. On the basis of current literature the OEO could represent a good compromise between potential

Table 1: Bacterial strains tested in this work.

Species	Strain	Origin	Reference	Species	Strain	Origin	Reference
*MRSA**	4	CF[1]	This work	*A. xylosoxidans**	1	CF[1]	This work
	5	CF[1]			21	CF[2]	
	6	CF[1]			34	CF[1]	
	9	CF[1]			39	CF[2]	
	11	CF[2]			41	CF[1]	
	12	CF[2]			42	CF[3]	
	13	CF[1]			43	CF[3]	
	15	CF[1]			45	CF[2]	
	16	CF[2]			48	CF[2]	
	17	CF[1]			49	CF[2]	
	20	CF[1]			50	CF[2]	
	23	CF[2]			52	CF[1]	
	24	CF[2]			53	CF[1]	
	25	CF[2]			54	CF[1]	
	26	CF			55	CF[1]	
	27	CF[1]			56	CF[1]	
	28	CF[2]			60	CF[1]	
	32	CF[1]			61	CF[2]	
	33	CF[3]			62	CF[1]	
					63	CF[1]	
*S. maltophilia**	1	CF[2]	This work	*S. maltophilia**	12	CF[1]	This work
	2	CF[1]			13	CF[1]	
	3	CF[1]			14	CF[1]	
	4	CF[2]			15	CF[1]	
	5	CF[1]			16	CF[1]	
	6	CF[1]			17	CF[1]	
	7	CF[2]			19	CF[2]	
	8	CF[1]			20	CF[1]	
	10	CF[1]			21	CF[2]	
	11	CF[1]			22	CF[2]	

* Strains isolated from CF patient (CF) at the Anna Meyer Children's Hospital (Florence, Italy). [1] sputum; [2] throat swab; [3] bronchial

antibacterial activity [8, 9, 11, 17] and tolerability [18] if properly formulated in specific drug delivery systems [19]. The aim of this work was to investigate the antimicrobial potential of *O. vulgare* EO, whose composition was previously determined [11], on a panel of 59 MDR strains belonging to three of the major groups of CF opportunistic pathogens (i.e. MRSA, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans*) estimating the bacteriostatic or bactericidal activity.

As a first step of the investigation, the molecular and/or phenotypic characterization of all the 59 representatives of the bacterial panel was performed revealing that the bacterial isolates of each species corresponded to different strains (data not shown). Then, the antibiotic resistance profile of each strain was determined using a panel of different antibiotics (Tables S1-S3). Most of the strains were resistant to multiple antibiotics (belonging to at least two major classes of antibiotics) and can be therefore defined as MDR according to the definition of the American Cystic Fibrosis Foundation [20]. In particular:

i) *A. xylosoxidans* strains (Table S1) showed a resistant rate higher than 60% for almost all categories of antibiotic tested: between β-lactams antibiotics only the combination piperacillin/ tazobactam, imipenem and meropenem showed lower resistance percentages. The resistant rates for colistin and minocycline were 62.5% and 50%, respectively. The percentage of antibiotics to which each strain was resistant ranged from 50% to 100%.

ii) *S. maltophilia* strains (Table S2) showed a resistant rate higher than 78% for β-lactams, aminoglycosides and fluoroquinolones antibiotics. The resistant rates for the combination trimethoprim/sulfamethoxazole and minocycline were 45% and 5.56%, respectively. The percentage of antibiotics to which each strain was resistant ranged between 50% and 100%.

iii) *Staphylococcus aureus* strains (Table S3) were highly resistant to β-lactams and macrolides. The resistant rate for fluoroquinoles and lincosamides was around 74% and 83% respectively, whilst the other rates were lower than 50% up to 0% for linezolid, tigecyclin, trimethoprim/sulphamethoxazole and vancomycin. One strain was resistant to teicoplanin. The percentage of antibiotics to which each strain was resistant ranged from 31.2% to 66.7%.

Subsequently, the antimicrobial activity of different concentrations of OEO was tested for each of the 59 strains listed in Table 1 by determining both MIC and MBC. Data obtained at 48 h of incubation in the presence of OEO are shown in Tables S1-S3 and summarized in Table 2. OEO exhibited antibacterial activity against all the 59 bacterial strains to a different extent.

Several MRSA and *A. xylosoxidans* strains had MIC of 0.50% and 0.25%, v/v, EO at 48 h of incubation. Results were consistent also at 24 h and 72 h (Table 2).

Quite interestingly, the analysis of MBC data revealed that the OEO had a strong bactericidal activity, which in most cases was consistent with the MIC values observed.

In general, OEO showed its inhibitory and microbicidal activity against human pathogens, even at low concentration: after 48 h, 0.5%, v/v, OEO was able to inhibit the growth of 100% of *A. xylosoxidans*, *S. maltophilia*, and MRSA, and to kill 100% of *S. maltophilia* and of MRSA and 95% of *A. xylosoxidans*.

In particular, the analysis of data from Table 2 revealed that after 48h:

i) *S. maltophilia* strains were more sensitive than those belonging to the other genera: no strain exhibited MIC and MBC higher than 0.125%, v/v. The lowest OEO percentages still active were 0.015% and 0.03, v/v, for MIC and MBC, respectively.

ii) *Achromobacter* strains were mostly inhibited and killed by OEO concentrations of 0.25% and 0.50%, v/v.

iii) MRSA had MIC and MBC values ranging from 0.5% to 0.125%, v/v, of OEO.

Data obtained in this work revealed that all the 59 tested strains were sensitive to OEO, even though to different extents. Overall the sensitivity of *S. maltophilia* strains was higher than that exhibited by MRSA and *A. xylosoxidans* strains. Moreover, there was no correlation between the MDR patterns and sensitivity to the OEO of strains belonging to the same species/group. Indeed, strains with a (very) different MDR profile exhibited a very similar degree of sensitivity to the OEO.

MBC values were in most cases completely consistent with the MIC values of the same strain and indicated that the OEO had a strong bactericidal activity on each of the 59 strains tested. Since the strains belong to very different bacterial species/genera (both Gram positive and Gram negative) the finding of such a broad activity of OEO might suggest that OEO has the same cellular target(s) on such widely different species. It is likely that the antibacterial activity of OEO is due to the combined effect of several bioactive molecules of the EO complex. Moreover, and quite interestingly, in spite of the large number of experiments carried out in this work, no mutant strain resistant to the EO tested was isolated. This finding strongly suggests the possibility that the antimicrobial activity of OEO is exerted toward multiple cellular targets. If this is so, the simultaneous blocking of the activity of different molecular targets should strongly decrease the probability of the appearance of a mutant able to resist the essential oils, as happens for most of the common antibiotics. For example, *S. aureus* is known for its involvement in CF and nosocomial infections and is frequently resistant to several antibiotics. OEO has been reported to reduce lipase and coagulase activity of *S. aureus* [21]. The lack of appearance of resistant strains to concentrations much lower than 2% OEO (maximum MBC value after 24 h) represents a very interesting finding that might pave the way to the use of OEO to fight the infections in CF patients.

Table 2: MIC and MBC in the twenty strains of *A. xylosoxidans*, *S. maltophilia* and MRSA.

EO concentration (% v/v)	MIC (mg/L) (O.D.)						MBC					
	24h		48h		72h		24h		48h		72h	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
A. xylosoxidans												
1.000	0	0	0	0	0	0	0	0	1	5	1	5
0.500	1	5	7	35	7	35	7	35	8	40	8	40
0.250	14	70	12	60	12	60	11	55	11	55	11	55
0.125	5	25	1	5	1	5	2	10	0	0	0	0
0.060	0	0	0	0	0	0	0	0	0	0	0	0
0.030	0	0	0	0	0	0	0	0	0	0	0	0
S. maltophilia												
1.000	0	0	0	0	0	0	0	0	0	0	0	0
0.500	0	0	0	0	0	0	0	0	0	0	0	0
0.250	1	5	0	0	0	0	3	15	0	0	0	0
0.125	7	35	9	45	9	45	12	60	5	25	5	25
0.060	3	15	0	0	1	5	4	20	6	30	4	20
0.030	8	40	10	50	9	45	1	5	9	45	11	55
0.015	0	0	1	5	1	5	0	0	0	0	0	0
0.007	1	5	0	0	0	0	0	0	0	0	0	0
MRSA												
1.000	0	0	0	0	0	0	0	0	0	0	0	0
0.500	5	26.3	5	26.3	5	26.3	7	36.8	5	26.3	5	26.3
0.250	8	42.1	8	42.1	8	42.1	7	36.8	8	42.1	8	42.1
0.125	6	31.6	6	31.6	6	31.6	5	26.4	6	31.6	6	31.6
0.060	0	0	0	0	0	0	0	0	0	0	0	0
0.030	0	0	0	0	0	0	0	0	0	0	0	0

Finally, the development of new inhaled antibiotics (or antimicrobial-like drugs) could be of great importance to improve the efficacy of the treatment of CF patients [22]. It is easy to imagine that an essential oil could be developed into an inhalation apparatus for use in patients with respiratory infections, as previously reported for basil, rosemary and eucalyptus [23-25]. Nevertheless, toxicological screenings are mandatory before suggesting a possible clinical use and possible additive/synergistic effects should be investigated since EO combinations and/or EOs plus antibiotics could boost their bactericidal effect, lowering the concentrations needed, and minimizing in turn the risk of side effects.

Experimental

Bacterial strains and growth conditions: The panel of 59 bacterial strains (20 for *S. maltophilia* and *A. xylosoxidans* and 19 for MRSA) tested in this work is reported in Table 1. The bacterial strains were isolated from different CF patients and each strain was maintained at -80°C under glycerol (25%, v/v) stock, and grown on Columbia blood agar (Thermo Scientific, Oxoid SpA, Strada Rivoltana, 20090 Rodano (MI) - Italy) at 37°C for 24 h.

Identification and typing of bacterial strains: Bacterial strains were identified using Matrix Assisted Laser Desorption Ionization Time-of-Flight (Maldi-Tof VITEK MS, bioMérieux Italia Spa, Italy).

Antibiotic resistance profiling: Susceptibility was evaluated to clinically-relevant antibiotics, specific for each pathogen [26-29] and selected across different antimicrobial families. Minimum Inhibitory Concentration (MIC) was evaluated for *S. maltophilia* by E-Test, and for *A. xylosoxidans* and MRSA by an automated system (Vitek 2, bioMérieux Italia Spa, Italy). Results were interpreted according to the available EUCAST (b) breakpoint tables or CLSI's (2012).

Origanum vulgare essential oil: The *O. vulgare* EO, extracted by steam distillation, was purchased from Prodotti Phitocosmetici Dott. Vannucci di Vannucci Daniela e C. Sas, Prato, Italy. The composition of the OEO used in this work has been already reported [11]. MIC and MBC were determined in TSB added with the EO in concentrations two-fold diluted from 2% to 0.007%, v/v, and the same volume of dimethylsulphoxide (DMSO, Carlo Erba Reagenti SpA, Milano, Italy), sterilized by filtration through filters with a pore diameter of 0.22 µm (Sartorius Italy Srl, Monza e Brianza, Italy).

Determination of MIC and MBC of O. vulgare EO: Determination of MIC, in broth micro-dilutions, was performed as described in standard protocols. Microtiter plates containing serial dilutions of the OEO were inoculated with aliquots of 100 µL of bacterial suspensions containing approximately 2×10^6 CFU/mL in a final volume of 200 µL. The negative control contained 200 µL of TSB, whereas the two positive controls contained 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; different antibiotics might be used according to the different resistance pattern of the tested bacteria [26-29]. Microplates were incubated at 37°C aerobically. After incubation, the Infinite 200 PRO multimode reader (Tecan), was used to detect density (using OD_{600}).

From each tube, at time "0" a 10 µL aliquot of the suspension was spread on TSA plates and incubated at 37°C aerobically; afterwards, the number of CFU was determined. The effect of OEO on bacteria was monitored at 24 h intervals up to 72 h by seeding 10 µL of the suspensions on TSA plates. Data obtained allowed the establishment of the bactericidal/bacteriostatic activity of OEO vs each organism, in terms of MIC, intended as the lowest concentration of OEO able to inhibit completely the growth of microorganisms in tubes and on plates. The MBC was defined as the concentration of OEO that killed at least 99.9% of the inoculum. All assays were performed in triplicate. Colony growth was verified even for each control. MIC and MBC were determined after incubation for 24, 48 and 72 h.

Statistical analyses: Means, standard deviations of bacterial counts and graphics were obtained through Microsoft Office Excel 2007 (Microsoft S.r.l., Milano, Italy).

Supplementary data: Details on the antibiotic resistance profile, *Origanum vulgare* essential oil MIC and MBC of each strain are also available.

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