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Susceptibility of methicillin-resistant staphylococci to oregano essential oil, carvacrol and thymol

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Abstract

The aim of this study was to evaluate the susceptibility of methicillin-susceptible and methicillin-resistant staphylococci (MSS, MRS) to oregano essential oil, carvacrol and thymol. The commercial aerial parts of *Origanum vulgare* L. were hydrodistilled and the essential oil analysed by gas- chromatography/electron impact mass spectrometry. The inhibition efficacy of this essence and its major components was assayed against 26 MSS and 21 MRS, using an agar dilution method. The methicillin resistance was thoroughly typed by Epsilometer test (E-test), polymerase chain reaction for *mec*A gene detection and PBP2′ latex agglutination test. The results clearly demonstrated that the comparison between the susceptibility of MSS and MRS to oregano oil, carvacrol and thymol showed no significant differences (Fisher's exact test, $P > 0.05$). The best minimum inhibitory concentration values were reported for carvacrol (0.015–0.03%, v/v) followed by thymol (0.03–0.06%, v/v) and oregano oil (0.06–0.125%, v/v).

Keywords: Oregano oil; Carvacrol; Thymol; Antibacterial activity; Methicillin resistance; Staphylococcus

1. Introduction

In recent years, the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) strains has been observed worldwide [1,2], often associated with extensive hospital outbreaks. Infections caused by methicillin-resistant staphylococci (MRS) have become a clinical and therapeutic problem because these organisms are resistant not only to β-lactams but also to many other antimicrobial agents. Resistance to semisynthetic penicillins (methicillin resistance) is frequently mediated by an additional penicillin-binding protein, PBP2′, encoded by the *mec*A gene, which has a very low affinity for β-lactam agents [3]. A characteristic of methicillin resistance is its heterogeneous nature, which can complicate conventional susceptibility testing since resistance may vary according to the testing conditions used [4] and therefore a combination of methods should be used.

Nasal and hand disinfectants are used to control the carriage and spread of MRS strains, but resistance is increasing and therefore alternative strategies or more effective agents are needed. An interesting approach to limit the transmission of these strains could be the use of essential oils as alternative agents or topical agents in the control of these harmful pathogens. In this regard, tea tree oil has already been explored as an alternative topical agent against MRSA [5,6]. Oregano is a very versatile plant and although it has been known for a long time, as a popular remedy, only now is it starting to be recognised for its potential therapeutic role such as diaphoretic, carminative, antispasmodic, antiseptic and tonic properties. Although it has a wide spectrum of antimicrobial activity, which has been the subject of several investigations in vitro [7–10] and in vivo [11–13], there is a lack of knowledge on its effectiveness on methicillin-resistant isolates of staphylococci.

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The aim of the present study was to evaluate the susceptibility of staphylococcal isolates, thoroughly characterised as MSS or MRS (by Epsilometer test, occurrence of the *mec*A gene and PBP2′), to oregano oil and its major components carvacrol and thymol. Furthermore, since methicillin resistance is often accompanied by resistance to other antimicrobial agents [3], the susceptibility profiles of MRS towards various chemotherapeutic antimicrobial agents were also evaluated.

2. Materials and methods

2.1. Characterisation of the strains

2.1.1. Identification

American Type Culture Collection (ATCC) standard strains used in this study were as follows: *S. aureus* ATCC 6538P, ATCC 25923 and ATCC 43300, *S. epidermidis* ATCC 12228. Furthermore, 43 strains of staphylococci isolated from respiratory tract and ocular infections and collected from outpatients in Italian hospitals were employed.

The identification of staphylococcal clinical isolates was conducted according to colony morphology, Gram staining, coagulase positivity and API Staph (API, Biomerieux). The strains were stored at −70°C in Microbanks™ and Singol beads were removed from the cryovials and used to directly inoculate Muller–Hinton broth (MH; Oxoid).

2.1.2. E-test for determining the minimum inhibitory concentrations (MICs) of oxacillin

The E-test® (PDM-Epsilometer, AB Biodisk, Solna, Sweden) was used to determine the MICs of oxacillin for 47 *Staphylococcus* spp. strains according to the manufacturer's reported methods. Breakpoint resistance at MIC ≥ 4.0 μg ml^{-1} for *S. aureus* and MIC ≥ 0.5 μg ml^{-1} for *S. epidermidis* was used [14].

2.1.3. Detection of the mec*A* gene by polymerase chain reaction (PCR) amplification

PCR amplification was carried out using two primers as described by Murakami et al. [15]. The two primers were: 5′-AAA ATC GAT GGT AAA GGT TGG C-3′ and 5′-AGT TCT GCA GTA CCG GAT TTG C-3′.

PCR was performed using Taq DNA polymerase (Qiagen) and related buffer. For amplification we used 100 ng of bacterial DNA (spectrometrically determined), 0.25 μM (each) primer, 200 μM dNTP mixture (Sigma), PCR 10× buffer providing a final concentration of 1.5 mM $MgCl_2$, Q-solution 5.0× and 0.5 μl of Taq DNA polymerase. DNA amplification was carried out for 40 cycles in 100 μl of reaction mixtures as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min with a final extension at 72°C for 5 min.

2.1.4. PBP2′ latex agglutination (LA) test

PBP2′ Latex Agglutination Test (Oxoid) was performed according to the manufacturer's instructions. The agglutination was observed visually and compared with a positive control (*S. aureus* ATCC 43300) and negative controls (*S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228).

2.1.5. Disc diffusion testing

Resistance of MRSA and MRSE strains to various antimicrobial agents was evaluated by disc diffusion testing according to the guidelines of the National Committee for Clinical Laboratory Standards [14] using discs (Oxoid) containing 2.5 μg trimethoprim, 5.0 μg ofloxacin, 10 μg gentamicin, 30 μg netilmicin, 10 μg chloramphenicol, 30 μg tetracycline, 15 μg erythromycin, 30 μg vancomycin.

2.2. Plant material and gas chromatography/electron impact mass spectrometry (GC/EIMS)

The aerial parts of *O. vulgare* L. obtained from a commercial source (A. Minardi, Ravenna, Italy) were subjected to hydrodistillation for 2 h using a modified Clevenger type apparatus and the essential oil was collected and stored at 4°C [16].

The GC and GC/EIMS analyses of the essence were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector, as previously described [17].

Carvacrol and thymol were purchased from Aldrich (Milan, Italy). Stock solution of 50% (v/v) thymol was prepared in dimethylsulfoxide (DMSO; BDH).

2.3. MICs

The MICs of oregano oil and its components carvacrol and thymol were determined by an agar dilution method according to the NCCLS guidelines [14] using a multipoint inoculator, delivering 0.3 μl of bacterial suspension (5×10^7 cfu ml^{-1}). The final concentration of essential oils in the medium ranged from 0.5% to 0.0035% v/v and DMSO maximum concentration was 1.0% (v/v). The plates were incubated at 37°C for 18–24 h and the MIC was defined as the lowest concentration of essential oil inhibiting visible growth. All determinations were performed in duplicate and two growth controls consisting of MH medium and MH with 1.0% (v/v) DMSO were included.

2.4. Statistical method

The MRS and MSS strains were compared with respect to MIC values to oregano oil, carvacrol and thymol using Fisher's exact test as statistical analysis.

Table 1
MICs of *O. vulgare* L., carvacrol and thymol for MRSA and methicillin-sensitive *S. aureus* strains typed by E-test, *mec*A PCR and LA test[a]

Isolate number *S. aureus*	E-test MICs of oxacillin (μg ml^{-1})	PCR *mec*A	LA test PBP2′	*Origanum* oil MIC (%, v/v)	Carvacrol MIC (%, v/v)	Thymol MIC (%, v/v)
1 (ATCC 43300)	16	+	+	0.125	0.015	0.06
2	>256	+	+	0.125	0.015	0.06
3	>256	+	+	0.125	0.03	0.06
4	>256	+	+	0.125	0.03	0.06
5	>256	+	+	0.125	0.03	0.06
6	>256	+	+	0.125	0.03	0.06
7	>256	+	+	0.125	0.03	0.06
8	>256	+	+	0.125	0.03	0.06
9	>256	+	+	0.125	0.03	0.06
10	4.0	−	−	0.125	0.03	0.03
11	2.0	−	−	0.06	0.015	0.03
12	1.5	−	−	0.06	0.015	0.03
13	1.0	−	−	0.06	0.015	0.06
14	1.0	−	−	0.125	0.015	0.06
15	1.0	−	−	0.125	0.03	0.06
16	0.75	−	−	0.125	0.03	0.06
17	0.5	−	−	0.125	0.03	0.06
18	0.38	−	−	0.06	0.03	0.03
19	0.38	−	−	0.125	0.015	0.06
20	0.25	−	−	0.125	0.03	0.06
21	0.25	−	−	0.125	0.03	0.06
22	0.19	−	−	0.125	0.03	0.06
23 (ATCC 6538P)	0.19	−	−	0.125	0.03	0.06
24 (ATCC 25923)	0.19	−	−	0.125	0.03	0.06
25	0.125	−	−	0.125	0.03	0.06

[a]+, positive result; −, negative result.

Table 2
MICs of *O. vulgare* L., carvacrol and thymol for MRSE and methicillin-sensitive *S. epidermidis* strains typed by E-test, *mec*A PCR and LA test[a]

Isolate number *S. epidermidis*	E-test MICs of oxacillin (μg ml^{-1})	PCR *mec*A	LA test PBP2′	*Origanum* oil (%, v/v)	Carvacrol MIC (%, v/v)	Thymol (%, v/v)
26	>256	+	+	0.125	0.03	0.06
27	>256	+	+	0.125	0.03	0.06
28	>256	+	+	0.125	0.03	0.06
29	>256	+	+	0.125	0.03	0.06
30	>256	+	+	0.125	0.03	0.06
31	>256	+	+	0.06	0.03	0.06
32	>256	+	+	0.125	0.03	0.06
33	>256	+	+	0.125	0.03	0.06
34	>256	+	+	0.06	0.015	0.03
35	>256	+	+	0.125	0.03	0.06
36	64	+	+	0.125	0.03	0.06
37	64	+	+	0.125	0.03	0.06
38	0.5	−	−	0.125	0.03	0.06
39	0.19	−	−	0.125	0.03	0.06
40	0.125	−	−	0.125	0.03	0.06
41	0.125	−	−	0.125	0.03	0.06
42	0.125	−	−	0.125	0.03	0.06
43	0.094	−	−	0.125	0.03	0.06
44	0.094	−	−	0.125	0.03	0.06
45	0.064	−	−	0.125	0.03	0.06
46 (ATCC 12228)	0.064	−	−	0.125	0.03	0.06
47	0.047	−	−	0.125	0.03	0.06

[a]+, positive result; −, negative result.

Table 3
Resistance of MRSA and MRSE strains to various antimicrobial agents according to the agar diffusion method[a]

Isolates	*n*	Number of resistant strains (%)							
		W	OFX	GEN	NET	C	TE	E	VA
MRSA	8	2 (25)	8 (100)	6 (75)	2 (25)	4 (50)	4 (50)	8 (100)	1 (12.5)
MRSE	12	1 (8.3)	3 (25)	7 (58.3)	0	2 (16.6)	3 (25)	3 (25)	0

[a]W, trimethoprim; OFX, ofloxacin; GEN, gentamicin; NET, netilmicin; C, chloramphenicol; TE, tetracycline; E, erythromycin; VA, vancomycin.

3. Results and discussion

The emergence of MRS strains as significant human pathogens and their growing prevalence in nosocomial infections increase the necessity to identify and to limit their spread. The detection of methicillin resistance in staphylococci is complex and difficulties exist in accurately identifying MRS mainly because the resistance is often heterogeneous and its expression is affected by different factors [3]. A number of methods have been studied to detect methicillin resistance in both *S. aureus* and *S. epidermidis*, but contradictory results have been reported [4,18,19]. In this study, most isolates (95.3%) showed complete concordance among E-test, *mec*A PCR (revealed by generation of a single 533-bp fragment) and PBP2′ (Tables 1 and 2). In accordance with previous reports [4,18,20] only two isolates with oxacillin MICs coincident with their breakpoints (4.0 μg ml^{-1} and 0.5 μg ml^{-1} for *S. aureus* and *S. epidermidis* respectively) were negative for *mec*A gene and PBP2′ detection. On the other hand, a complete agreement between PCR and PBP2′ was found for all isolates, a confirmation of the reliability of the LA test for PBP2′ detection also in *S. epidermidis* although it is recommended for *S. aureus* [21,22]. As shown in Table 3 the MRS isolates showed different susceptibility patterns when they exhibited multidrug resistance. A very high resistance to fluoroquinolone/macrolide and to gentamicin was detected for MRSA and MRSE respectively.

All *S. aureus* and *S. epidermidis* strains were susceptible to oregano oil, carvacrol and thymol with no statistically significant difference between MRS and MSS strains (Fisher's exact test, $P > 0.05$). Interestingly this susceptibility was independent of drug susceptibility patterns. In line with previous works [7–9,11] our data also confirm the relevant antimicrobial activity of carvacrol and thymol in the oregano oil. The best MIC values were reported for carvacrol (0.015–0.03%, v/v), followed by thymol (0.03–0.06%, v/v) and oregano oil (0.06–0.125%, v/v). The higher MIC values of oregano oil compared to its components can be related to the fact that carvacrol and thymol represent only a fraction of the whole essence (38.7% of the total oil). The qualitative and quantitative composition of oregano oil analysed by GC/EIMS is shown in Table 4. The essential oil is characterised by principally phenol constituents, carvacrol and thymol (Fig. 1) and by their two precursors monoterpene hydrocarbons, γ-terpinene and *p*-cymene (11.7 and 14.6% of the total oil, respectively). No antimicrobial activity has been reported for *p*-cymene and γ-terpinene [5,7–9]. Probably, the lack of activity of these hydrocarbon monoterpenes is due to the absence of the phenolic hydroxyl group [8]. On the other hand, even if oregano oil had MIC values slightly higher than its components, it could be advantageous to use as a whole because some of its major compounds can play an

Table 4
Composition of *O. vulgare* L. essential oil as determined by GC/EIMS

Retention index	Compound	%
933	α-thujene	1.5
941	α-pinene	0.9
955	camphene	0.4
979	1-octen-3-ol	1.6
989	3-octanone	2.4
993	myrcene	1.6
995	3-octanol	1.6
1006	α-phellandrene	0.3
1013	3-carene	0.1
1020	α-terpinene	2.4
1027	*p*-cymene	14.6
1033	limonene	0.4
1034	β-phellandrene	0.2
1036	1,8-cineole	0.4
1064	γ-terpinene	11.7
1070	*cis*-sabinene hydrate	0.2
1089	terpinolene	0.2
1101	linalool	0.3
1145	camphor	0.2
1170	borneol	0.9
1178	4-terpineol	1.3
1185	*p*-cymen-8-ol	0.3
1194	*cis*-dihydrocarvone	0.1
1196	α-terpineol	0.2
1233	methyl thymol	4.5
1242	methyl carvacrol	8.4
1292	thymol	24.7
1299	carvacrol	14.0
1356	thymol acetate	0.2
1371	carvacrol acetate	0.1
1386	β-bourbonene	0.2
1420	β-caryophyllene	0.5
1478	γ-muurolene	0.2
1482	germacrene D	0.2
1504	(*E*,*E*)-α-farnesene	0.1
1506	β-bisabolene	1.6
1511	*cis*-γ-cadinene	0.1
1514	*trans*-γ-cadinene	0.4
1578	spathulenol	0.2
1685	*epi*-α-bisabolol	0.3
	Yield	0.64

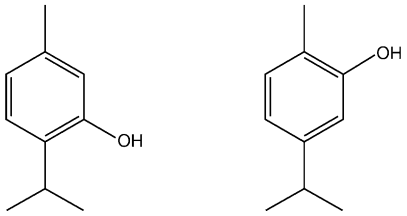

Fig. 1. Structure of carvacrol and thymol.

important therapeutical role with additional antioxidant or antispasmodic activities [23].

In conclusion, the findings of the present study indicate that oregano oil, in addition to other properties, has a potential as a topical antibacterial agent against MRS strains.

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