Essential oil antibacterial activity against methicillin-resistant and -susceptible \textit{Staphylococcus aureus} strains

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Abstract

\textit{Staphylococcus aureus} is a pathogen causing infections that range from skin lesions to life threatening conditions. Methicillin resistance development in \textit{S. aureus} strains represents a huge problem worldwide. The inhibition efficacy of twelve different essential oils (laurel, anise, oregano, basil, lavender, mint, rosemary, tea tree, bergamot, grapefruit, ginger and winter savory) and of the antibiotic Vancomycin was tested against \textit{S. aureus} NCTC6571 and clinical isolates using paper disk diffusion assay and broth microdilution test methods. Forty-four \textit{S. aureus} strains isolated from different human sample were characterized for antibiotic resistance and 41% of them were methicillin resistant. Among the twelve tested oils basil, oregano and savory showed stronger inhibition effect on \textit{S. aureus} growth than Vancomycin. These results can be useful for the formulation of topical gel containing selected essential oils active against \textit{S. aureus} strains.

Introduction

\textit{Staphylococcus aureus} is an important cause of sepsis and one of the main nosocomial pathogens; its infections have often been associated with significant morbidity and mortality. In the pre-antibiotic era, blood infections due to \textit{S. aureus} resulted in an 80% mortality rate; although nowadays the prognosis has improved, the impact of the disease remains dramatically high. Recent studies have estimated that the hospital mortality rate, for patients with infections from Methicillin-Resistant Strains (MRSA), is around 30%, with peaks of 65% in some centers. In general, the mortality rate due to \textit{S. aureus} infections is higher than that caused by HIV virus, viral hepatitis, tuberculosis and influenza. In the human population, approximately 20-25% of individuals are constantly infected, while the remaining part is less frequently contaminated. \textit{S. aureus} is therefore by far, one of the most important pathogens in bacterial infections, although it is part of the normal human microflora.

Several factors, such as the alterations of both congenital (e.g. Down syndrome) and acquired (e.g. diabetes mellitus, rheumatoid arthritis) leukocyte chemo-taxis, alterations of antibody and of the intracellular bacteria killing after phagocytosis, due to the inability to activate the membrane-related oxidative system, predisp...
Materials and Methods

Microorganisms

*S. aureus* strains were collected by the Microbiology Departments of SS. Antonio and Biagio e Cesare Arrigo Hospital (Alessandria, Italy), of the Città di Alessandria Clinic (Alessandria, Italy) and of the Cardinal Massaia Hospital (Asti, Italy). Forty-four clinical strains of *S. aureus*, including 17 methicillin-resistant, were isolated from: bronchial aspirate (4 strains), cutaneous swab (13 strains), throat swab (3 strains), blood culture (2 strains), eye swab (1 strain), heel swab (1 strain), nasal swab (4 strains), pacemaker pocket swab (1 strain), pus swab (1 strain), urine culture (1 strain), vaginal swab (1 strain), wound swab (10 strains), ulcer swab (1 strain), tracheal aspirate (1 strain). All the strains were identified using the VITEK® 2 automated system (bioMerieux, France).

Essential Oils (EOs)

The employed EOs were extracted from *Laurus nobilis* L. (laurel), *Pimpinella anisum* L. (anise), *Thymus capitatus* L. (oregano), *Ocimum basilicum* L. (basil), *Lavandula latifolia* Medik (lavender), *Mentha spicata* L. (mint), *Rosmarinus officinalis* L. (rosemary), *Melaleuca alternifolia* Cheel (tea tree), *Citrus bergamia* Risso & Poit (bergamot), *Citrus paradisi* Macfad (grapefruit), *Zingiber officinale* Roscoe (ginger) and *Satureja montana* L. (winter savory), all provided by Flora s.r.l. (Lorenzana, Pisa, Italy).

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Gas Chromatography/Mass Spectrometry (GC/MS) analyses were performed as previously detailed in Massa et al (2018).11 Briefly, a Gas Chromatograph PerkinElmer Clarus 500 GC/FID/MS equipped with non-polar capillary column HP-5MS (5% diphenyl, 95% dimethylpolysiloxane), with a length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 mm, was used. Helium flow was 1.5 ml min⁻¹. Analyses were performed in the temperature range 60–280 °C, and the heating time was 10 °C min⁻¹. The injection volume was 1 ml of 1:50 (v/v) solution of each EOs in dichloromethane. The analysis was repeated three times for each sample.

Minimal Inhibitory Concentration (MIC) of antibacterial agents

The Minimal Inhibitory Concentration (MIC) of the fifteen antibacterial drugs [Benzypenicillin (BPC), Oxacillin (OXA), Gentamicin (GEN), Levofloxacin (LVX), Erythromycin (ERY), Clindamycin (CLI), Linezolid (LZD), Daptomycin (DAP), Teicoplanin (TEC), Vancomycin (VAN), Tetracycline (TET), Tigecycline (TGC), Fusidic Acid (FUS), Rifampicin (RIF), Trimethoprim-sulfamethoxazole (SXT)] were measured by VITEK® 2 AST card using VITEK® 2 automated system (bioMerieux, France). *S. aureus* NCTC 6571 strain was used as reference strain. Briefly, strain suspensions obtained in physiological solution were adjusted to 0.5 McFarland by measuring absorbance at 600 nm. These suspensions were then loaded into the instrument in VITEK® 2 AST cards that provided a series of antibiograms and tests for the detection of resistance (ESBL, cefoxitin screen, high level aminoglycoside resistance, inducible clindamycin resistance).

Disk diffusion assay

The following assays were carried out with 44 *S. aureus* strains and the reference strain *S. aureus* NCTC 6571. Vancomycin antibacterial effects were evaluated according to EUCAST Disk Diffusion Method for Antimicrobial Susceptibility v. 7.0 (January 2019). The sensitivity to the EOs was assessed using agar disk diffusion method: strain suspensions (0.5 McFarland), obtained in physiological solution, were swabbed on Mueller Hinton Agar (Biolife Italiana s.r.l., Italy) plates. Filter paper disc (6.0 mm diameter) were placed on the agar surface and added with 10 µl of pure EO. Pure dimethyl sulfoxide (DMSO) (D-8418 - Sigma-Aldrich, St. Louis, MO, USA) (10 µl) and organic linseed oil (10 µl) disks were used as negative controls, while vancomycin was considered as positive control. Plates were incubated at 37 °C for 24 h. All experiments were performed in triplicate. The halos were measured in mm using calipers.

Minimal Inhibitory Concentration (MIC) of EOs

MIC of the EOs with a sensitivity test (disk diffusion assay) higher than Vancomycin were determined using EUCAST Method with some modifications. Briefly, EOs were dissolved in DMSO 20% (final well of each titer without EO in which all the cells are metabolically active). The chemical composition (%) of the fifteen antibacterial drugs were measured by gas-chromatography analysis, is reported in Supplementary Table 1, also reported in Massa et al. (2018).11 Blue lines underline the common

Fluorescent Diacetate Assay (FDA)

Fluorescent Diacetate Assay (FDA) (Sigma-Aldrich, St. Louis, MO, USA) stock solution (2.6 M) was prepared solving 1 g of fluorescent diacetate in 1 ml of sterilized potassium-phosphate buffer (8.7 g KH2PO4 and 1.3 g KHPO4 in 1 l deionized water). Bacterial cells were treated as reported for the MIC determination. The microtiter plates were incubated at 37 °C for 24 h. After incubation, 40 µl of 2.4 mmol l⁻¹ FDA were added to cell suspension to a total volume of 240 µl per well and incubated in the dark at 37 °C up to 60 min. Fluorescence intensity was measured in a TECAN microplate reader Infinite F200 pro (Tecan, Switzerland) using 492 nm excitation and 510 nm emission filters. The percentage of Fluorescence Inhibition (%FI) was calculated using the equation from Machado and Soares (2013): %FI = 100 – [(F/Fmax) × 100] where F is the fluorescence of the assay (cells treated with EOs) and Fmax is the mean fluorescence of the positive controls (final well of each titer without EO in which all the cells are metabolically active).

Statistical analysis

Statistical analyses were performed using StatView 4.5 (Abacus Concepts, Berkeley, CA, USA); data were compared by one-way ANOVA, followed by a post-hoc PLSD test (p<0.05).

Results

The 45 clinical strains of *S. aureus*, were listed in Table 1, also reporting their isolation origin and their response to different antibiotic drugs (MIC results). Following the interpretation of Cefoxitin screening, 38% (17 strains) of isolated *S. aureus* resulted MRSA. According to The European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameters (Version 9.0, 2019), the 100% (44 strains) of isolated *S. aureus* resulted to be resistant to SXT, 88.6% (39 strains) to BPC, 49.9% (18 strains) to OXA or to LVX, 38.6% (17 strains) to ERY, 13.6% (6 strains) to GEN, 6.8% (3 strains) to CLI, 4.5% (2 strains) to DAP or to TET, 2.3% (1 strain) to TEC, to VAN, to FUS or to RIF. Finally, none of the isolated strains resulted to be resistant to LZD or to TGC.

Disk diffusion assay

The chemical composition (%) of the twelve EOs, obtained by gas-chromatography analysis, is reported in Supplementary Table 1, also reported in Massa et al. (2018).11 Blue lines underline the common
components of winter savory and oregano EOs: all the reported chemical components occurring in winter savory, with the exception of Linalyl acetate, were also present, even if in different concentrations, in the oregano EO.

The results of disk diffusion assay performed on the clinical strains of *S. aureus* and on the NCTC 6571 reference strain are shown in Figure 1. The individual data related to the negative controls carried out with DMSO and linseed oil have not been reported as no strains have been inhibited.

In general, most of the oils were effective on a considerable number of strains, with a trend that did not allow to highlight differences in the sensitivity of meticillin-resistant strains compared to the others (data not shown). For each EO, strains that recorded a final value greater than Vancomycin were considered significant and the subsequent tests for determining the minimum inhibitory concentration and evaluation of the metabolic activity were then carried out on them and the relative oils. Figure 1 shows the presence of an important inhibitory action on *S. aureus* by oregano and winter savory EOs, which have proved to be active on all the strains considered, with a peak of about 300% of inhibition compared to van-

| Sample                     | Strain      | Cefoxitin    | MRSA*         | BPC     | OXA*       | GEN         | LVX              | iR# to Clindamycin |
|---------------------------|-------------|--------------|---------------|---------|------------|--------------|-------------------|--------------------|
| Reference strain NCTC 6571| Negative    | 0.12         | 0.5          | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Cataro swab 19            | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Bronchial aspirate 20     | Positive     | MRSA >0.0     | 4            | Negative | >0.5       | 2             | 0.25             | <=0.5             |
| Cataro swab 21            | Negative    | >0.5         | <=0.5       | <=0.5   | <=0.12     | Negative     | >0.5             | 2                 |
| Cataro swab 22            | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 1                 | 2                 |
| Cataro swab 23            | Negative    | 0.25         | <=0.5       | 8       | 0.25       | Negative     | >0.5             | 2                 |
| Cataro swab 28            | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Negative     | 1                 | <=0.5             |
| Cataro swab 31            | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Bronchial aspirate 39     | Negative    | >0.5         | <=0.5       | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Throat swab 40            | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Wound swab 41             | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Blood culture 53          | Positive     | MRSA >0.5     | <=0.5       | 4       | Positive     | MLS >0.5     | >0.5             |
| Throat swab 54            | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Cataro swab 61            | Negative    | >0.5         | 0.5         | <=0.5   | >0.5       | Positive     | MLS >0.5          |
| Cataro swab 62            | Negative    | >0.5         | >0.5        | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Nose swab 100             | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 0.5              | <=0.5             |
| Cataro swab 101           | Negative    | 0.06         | 0.5         | <=0.5   | 0.25       | Negative     | 0.5              | <=0.5             |
| Nasal swab 102            | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 0.5              | <=0.5             |
| Urine culture 112         | Negative    | >0.5         | 2           | <=0.5   | 0.5        | Negative     | 1                 | <=0.5             |
| Wound swab 113            | Negative    | 0.16         | <=0.5       | <=10    | <=0.5      | Negative     | 1                 | <=0.5             |
| Vaginal swab 114          | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Throat swab 115           | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Nasal swab 116            | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Parakeratin pocket swab 141| Negative   | >0.5         | <=0.5       | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Wound swab 142            | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Wound swab 143            | Negative    | <=0.5        | <=0.25      | <=0.5   | <=0.12     | Negative     | 0.5              | <=0.5             |
| Fist swab 144             | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Nasal swab 145            | Negative    | >0.5         | <=0.5       | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Eye swab 146              | Positive     | MRSA >0.5     | >0.5       | 4       | Positive     | MLS >0.5     | 0.25             |
| Wound swab 147            | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | >0.5             |
| Wound swab 182            | Negative    | >0.5         | 0.5         | <=0.5   | 1           | Negative     | 0.5              | <=0.5             |
| Wound swab 183            | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Urect swab 184            | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Blood culture 185         | Negative    | >0.5         | <=0.25      | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Wound swab 187            | Negative    | >0.5         | <=0.5       | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Tracheal aspirate 188     | Positive     | MRSA >0.5     | >0.5       | 0.5     | Positive     | MLS >0.5     | >0.5             |
| Wound swab 189            | Negative    | <=0.5        | <=0.25      | 4       | 0.5         | Positive     | MLS >0.5          |
| Wound swab 190            | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Positive     | MLS >0.5          |
| Nasal swab 191            | Negative    | <=0.5        | <=0.25      | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Cataro swab 223           | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Negative     | 1                 | <=0.5             |
| Cataro swab 224           | Positive     | MRSA >0.5     | >0.5       | >0.5    | >0.5       | Negative     | 1                 | <=0.5             |
| Cataro swab 231           | Negative    | >0.5         | 0.5         | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Cataro swab 232           | Negative    | 0.06         | 0.5         | <=0.5   | 0.25       | Negative     | 1                 | <=0.5             |
| Cataro swab 233           | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |
| Cataro swab 234           | Negative    | >0.5         | 0.5         | <=0.5   | <=0.12     | Negative     | 1                 | <=0.5             |

**Table 1. Characterization of the response to different antibiotic drugs in one reference strain and in the forty-four clinical strains of *S. aureus*.

RGC: Benzylpenicillin; OA: Oxacillin; GEN: Gentamicin; LVX: Levofloxacin; ERY: Erythromycin; CLI: Clindamycin; LZD: Linezolid; DAP: Daptomycin; TEC: Teicoplanin; VAN: Vancomycin; TET: Tetracycline; TGC: Tigecycline; FUS: Fusidic Acid; Rif: Rifampicin; SST: Streptothricin-sulfathiazole; *: Interpretation of Cefoxitin screening and Oxacillin MIC values to determine MRSA strains according to EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance, Version 1.0 December 2013; # Inducible Resistance to Clindamycin; § Interpretation of Inducible resistance to Clindamycin. [Microbiology Research 2019; 10:8331] [page 33]**
comycin. In particular, 32% of the strains proved to be sensitive to winter savory EO (data not shown) with an efficacy of 250%, while 50% of them showed a value of less than 200%. Oregano oil instead inhibited 50% of the strains with an efficacy of 200%, and only 23% were below this threshold. Lavender, basil and tea tree oils were effective against at least 50% of the tested strains, despite their inhibitory effect being lower than that of oregano and winter savory and are closer to that of positive control (vancomycin). The mint EO can be considered a special case: in fact, it inhibited very strongly the growth of three strains, with percentages higher than 300%, while for 40% of strains, this oil induced an effect comparable to those of oregano and winter savory. Laurel, rosemary and grapefruit EOs had a significant effect on 28%, 19% and 9% of the strains, respectively, while none of them was sensitive to the action of anise, bergamot and ginger oils.

**Minimal Inhibitory Concentration (MIC)**

The results obtained for each oil from the analysis of MIC on the *S. aureus* strains considered are reported in Table 2.

Winter savory EO showed an excellent activity, inhibiting *S. aureus* growth at concentrations of 0.125% and 0.25% v/v, respectively for 43% and 52% of the strains, with only two cases where it drops to 0.062% v/v.

Oregano EO instead presented MIC of 1% v/v for more than 40% of the tested strains, while all the others were sensitive to lower concentrations, respectively of 0.5% and of 0.25% v/v for 26% and 30% of cases. On the contrary, basil and mint oils showed higher MIC values, between 2 and 4% v/v, although for basil, 44% of the strains needed a concentration higher than 4% v/v.

Although lavender EO was effective on a greater number of strains if compared to Tea Tree, it presented MIC of 2% v/v for 65% of them, while Tea Tree EO inhibited growth at a concentration of 1% v/v in 77% of the cases. Finally, only two strains were sensitive to lavender EO for concentrations of 0.5 and 0.25% v/v. Grapefruit EO was fairly effective, with concentrations lower than or equal to 0.125% v/v, while rosemary stabilized at MIC values of 2% v/v. Finally, laurel EO showed MIC higher or equal to 4% v/v. In general, as already found in the disk diffusion assay, the MRSA strains, shown in grey in table 2, did not have significant differences in MIC values compared to non-methicillin-resistant strains.

**Fluorescein Diacetate Assay (FDA)**

FDA is hydrophobic, colorless, and non-fluorescent. It diffuses freely into

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**Table 2. The results obtained for each oil from the analysis of MIC on the *S. aureus* strains considered.**

| *S. aureus* | Laurel | Oregano | Basil | Lavender | Mint | Rosemary | Tea Tree | Grapefruit | Winter savory |
|-------------|--------|---------|-------|----------|------|----------|----------|------------|---------------|
| NCTC 6571   |        |         |       |          |      |          |          |            |               |
| 19          | 0.5    | >4      | 2     | 1        | 1    | 0.005    | 0.125    | 0.02       |
| 20          | 1      | >4      | 1     | 1        | 1    | 0.125    | 0.125    |            |
| 21          | >4     | 0.5    | 2     | 2        | 1    | >4       | 0.125    | 0.125      |
| 22          | 0.5    | 1       |       | 1        | 1    | 1        | 0.125    |            |
| 23          | 1      | >4      | 1     | 1        | 1    | 0.125    | 0.125    |            |
| 24          | 1      | >4      | 1     | 1        | 1    | 0.125    | 0.125    |            |
| 25          | >4     | 0.5    | 1     | 1        | 1    | 1        | 0.125    |            |
| 26          | >4     | 1      | 2     | 4        | 1    | 1        | 0.125    |            |
| 27          | 1      | >4      | 1     | 1        | 1    | 1        | 0.125    |            |
| 28          | >4     | 0.5    | 1     | 1        | 1    | 1        | 0.125    |            |
| 29          | 0.25   | >4      | 2     | 1        | 1    | 1        | 0.125    |            |
| 30          | 0.5    | 1       | 2     | 4        | 1    | 1        | 0.125    |            |
| 31          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 32          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 33          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 34          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 35          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 36          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 37          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 38          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 39          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |
| 40          | >4     | 0.25   | 1     | 1        | 1    | 1        | 0.125    |            |

**Figure 1.** Disk diffusion assay was carried out with 44 *S. aureus* strains and the reference strain *S. aureus* NCTC 6571. Vancomycin antibacterial effects were evaluated according to EUCAST Disk Diffusion Method for Antimicrobial Susceptibility v. 7.0 (January 2019). The sensitivity to the EOs was assessed using agar disk diffusion method.
undamaged cells and is hydrolysed into a more polar yellow–green fluorescent product (fluorescein) and two acetate molecules. Figure 2 shows the results for the reduction of the metabolic activity induced by decreasing concentrations of the different EOs, expressed as the mean values of data obtained for all the tested strains.

While for Laurel, Tea Tree, Rosemary and Lavender EOs, the reduction in metabolic activity was at least 50% at maximum concentration (4%), this was drastically reduced to 0 starting from the sub MIC concentrations. On the contrary, oregano and winter savory retained levels of reduction of metabolic activity that approximate to 10% even at the lowest concentrations. A similar trend was observed for mint and basil EOs, although the last stabilized on lower final value and both were less effective at intermediate concentrations.

**Discussion**

EOs antibacterial activities are documented in several studies present in literature. Considering the current and still increasing problem of drug resistance, also referring to MRSA strains diffusion, the antimicrobial properties of the essential oils can be considered as a precious source of natural formulations. The results obtained in the present work are encouraging to the possible practical use of more effective EOs in inhibiting the growth and activity of *S. aureus*; moreover, considering the number of tested strains, they add important statistical data to those already present in the literature. Although the deepening of the mechanisms of action can be useful in the research for new molecules for therapeutic use, it is known that the synergistic mechanisms that exist between the components of essential oils are important in determining their effects. In this sense, the choice to directly compare the action of essential oils with that of the most used antimicrobial molecules in the clinical field was considered a suitable approach to establish their effectiveness. The data
obtained indicate that significant differences in the efficacy of EOs towards the 45 (44 clinical isolates and one reference strain) considered S. aureus strains, exist. However, these responses did not depend on the resistance to methicillin, which characterizes 39% of the tested strains: in fact, a significant difference in the efficacy of the EOs towards the resistant and sensitive methicillin strains was never observed. This information is in agreement with various studies concerning the susceptibility of MRSA to Tea Tree EO, which was not dependent on methicillin-sensitive organisms.15,16 Also, the work carried out by Chao and coworkers17 demonstrate the efficacy of many EOs on MRSA strains, suggesting that they could also be exploited in the treatment of infections aggravated by antibiotic resistance.

Among the twelve tested EOs in our work, oregano and winter savory EOs were excellent in their antibacterial effect, as they were effective on all the S. aureus strains, in accordance to previous studies.18-20 Similar results were obtained with mint EO.17,21-23 MIC results indicated oregano and winter savory EOs as more effective towards S. aureus, with concentrations lower or equal to 1% v/v and 0.25% v/v, respectively. In particular, these values confirm what was found for oregano oil by Chedia et al.,19 which reported MIC values ≤0.05% v/v. Comparable concentrations were verified only in the case of grapefruit, which is however active on a limited number of strains, in line with the values ≤1% v/v reported for S. aureus by Adukwu et al.24 For lavender EO, MIC values only slightly higher than those of oregano and winter savory were measured, but generally higher than the value of 0.32% v/v registered by Inouye et al.25 Also for tea tree EO, MIC less than or equal to 1% v/v are reported in the literature,26,27 although in the present work some strains proved to be sensitive only at a concentration of 2% v/v. On the other hand, data relating to laurel, basil, mint and rosemary EOs, show higher MIC values, and their use for the formulation of an effective mixture is therefore based above all on the evaluation of the ability to reduce metabolic activity at lower concentrations. The experimental data obtained from the analysis of the metabolic activity of the microorganisms provide new information with respect to the literature, since most of the works are limited to screening by disk diffusion test, to the evaluation of MIC values, and to the analysis of the constituents present in greater quantities. The reduction values of the metabolic activity with respect to the controls, obtained after the treatment with EOs, proved effective, indicate that they act differently. This observation could be linked to the presence of specific mechanisms of action of each oil, which in turn depend on the chemical constituents and the effects that are determined by their interaction. In the case of S. aureus, the trend in the reduction of metabolic activity due to the treatment with essential oils is comparable for the concentrations tested: oregano, winter savory, basil and mint proved to be effective even at low concentrations, while tea tree, laurel, lavender and rosemary need higher concentrations to achieve comparable effects.

Considering the large number of different chemical compounds present in EOs, it is very likely that the antibacterial and antifungal activities are not attributable to a single mechanism of action, but that result from the effect of the various constituents on different cellular targets.26 The cytotoxicity of EOs is linked to one of their important characteristics, hydrophobicity, which allows it to diffuse bacterial and eukaryotic cells through the wall and the cytoplasmic membrane. In fact, it seems that the oils interact with the membranes differently depending on the structure and the physicochemical properties of their components, altering the functions of various molecular structures. This may result in changes in the lipid bilayer, with variations in membrane fluidity, degradation of the cell wall and damage to membrane proteins: in particular transport systems, enzymes, ion channels and receptors.28,29 Although a certain amount of damage can be tolerated by bacterial cells without an effective reduction of vitality, massive alterations of cellular content and the loss of fundamental molecules can lead to death.30 In bacteria, permeabilization of membranes is mainly associated with ion loss, reduction of membrane potential, collapse of the proton pump and depletion of ATP reserve. EOs can also coagulate the cytoplasm or damage lipids and proteins, with the loss of macromolecules and cell lysis.31,32

EOs are, by definition, complex mixtures of many different molecules from the chemical point of view as reposted in supplemental table. It is therefore spontaneous to ask whether the properties and biological effects attributed to them are only the result of the action of the components present at the highest concentrations, or whether they reflect their more complex interaction. The interactions between the components of EOs can produce four types of effects: indifferent, additive, antagonist or synergistic.33 The synergy in particular, or synergistic effect, is observed when the combined effect of two or more substances is greater than the sum of their individual effects. Some studies have concluded that EOs in their entirety have a greater antibacterial activity compared to mixtures formed by the components present in greater quantities.14 This suggests the fundamental importance of minor components, which can exert a synergistic type effect by enhancing the final result. Actually, the main components often reflect the biological characteristics of the EOs they come from,34 but their activity can be modulated by the minor ones. Together, the various components play a fundamental role in defining the fragrance, density, color and all the physical characteristics of EOs, but they are especially important in ensuring penetration into cells and fixation to the wall and membranes.10

Although few studies have investigated the effects of EOs and/or their components in combination, some mechanisms of antimicrobial action are linked to synergistic interactions. They include the inhibition of common biochemical pathways and protective enzymes, as well as the use of active agents in the cell wall in order to increase the entry of other antimicrobials.35 Bassolé et al.36 summarized what is known about the antimicrobial efficacy of EOs and their components tested in combination. In basil EO, for example, the grea-test antimicrobial activity has been attributed to two components, eugenol and linalool, and a synergistic effect has been highlighted.37 The basic idea is the use of these data for the visualization of a mixture exploitable in the development of a medical device for external use, which allows the prevention, as well as the treatment, of any infections. The positive fact is that the formulated mixtures were effective in 100% concentration tests compared to traditional antimicrobials. Clearly it is necessary to implement the studies to arrive at defining a final concentration that is satisfactory from the point of view of biological activity, but which also allows to support the production costs of a medical device.

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