# Essential oil antibacterial activity against methicillin-resistant and -susceptible *Staphylococcus aureus* strains

**Elisa Bona,[1] Nadia Massa,[2] Giorgia Novello,[2] Matteo Pavan,[2] Andrea Rocchetti,[3] Graziella Berta,[2] Elisa Gamalero[2]**

**[1]Dipartimento di Scienze ed Innovazione Tecnologica, Università del Piemonte Orientale, Vercelli, Italy**
**[2]Dipartimento di Scienze ed Innovazione Tecnologica, Università del Piemonte Orientale, Alessandria, Italy**
**[3]Azienda Sanitaria Santi Antonio, Biagio e Cesare Arrigo, Alessandria, Italy**

## Abstract

*Staphylococcus aureus* is a pathogen causing infections that range from skin lesions to life threatening conditions. Methicillin resistance development in *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 *S. aureus* NCTC6571 and clinical isolates using paper disk diffusion assay and broth microdilution test methods. Forty-four *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 *S. aureus* growth than Vancomycin. These results can be useful for the formulation of topical gel containing selected essential oils active against *S. aureus* strains.

## Introduction

*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 *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%,[1] with peaks of 65% in some centers.[1] In general, the mortality rate due to *S. aureus* infections is higher than that caused by HIV virus, viral hepatitis, tuberculosis and influenza.[2] In the human population, approximately 20-25% of individuals are constantly infected, while the remaining part is less frequently contaminated.[3] *S. aureus* is therefore by far, one of the most important pathogens in bacterial infections, although it is part of the normal human microflora.[4]

Several factors, such as the alterations of both congenital (*e.g.* Down syndrome) and acquired (*e.g.* diabetes mellitus, rheumatoid arthritis) leukocyte chemiotaxis, alterations of antibody and of the intracellular bacteria killing after phagocytosis, due to the inability to activate the membrane-related oxidative system, predispose the onset of infections by *S. aureus*. Other common predisposing factors include: i) the presence of skin lesions or foreign bodies (*e.g.* prostheses, intravenous devices); ii) the presence at the same time of infections sustained by other agents, in particular viruses, or of chronic diseases such as neoplasia and heart disease; and iii) the antibacterial use for prophylactic or therapeutic purposes.[5]

The presence of *S. aureus* in the nostrils has long been considered as one of the main risk factors for the development of infections and bacteremia.[6] Skin infections include folliculitis and impetigo, but also boils that can involve the subcutaneous tissues causing the onset of symptoms such as fever.

Isolation of *S. aureus* from cultures such as pus, blood, cerebrospinal fluid should always be considered clinically significant; however, the simple colonization is not considered sufficient to initiate a therapy, except when the patient is infected with a MRSA strain: in this case the decolonization is part of a specific infection control policy.[7] At present, no vaccines are available for the prevention of *S. aureus* diseases. Hospital-wide infection control measures should be considered critical in preventing nosocomial infections, especially for MRSA.[8] The emergence and the increasing spread of drug-resistant pathogens are significant health problems that impose a certain urgency towards research and the possible application of new drugs with high therapeutic efficiency.

In this context, the natural products, such as Essential Oils (EOs), are increasingly protagonists in the field of traditional medicine, exhibiting antibacterial and antifungal properties known for centuries among the folk remedies. EOs are natural complex formulation of organic compounds, characterized by a strong fragrance; they rarely present color, are liposoluble and soluble in organic solvents. They are produced by aromatic plants as secondary metabolites and are extracted from flowers, leaves, seeds, fruits, stems, buds, roots, wood and bark.[9] In nature, EOs are essential for plants thanks to their antibacterial, antiviral, antifungal, and also play an important protective action against herbivores, reducing the palatability of the plant.[10] Over the years, EOs have gained interest as potential sources of natural bioactive molecules for the treatment of infections and diseases. In medicine, they are known for their antiseptic, antibacterial, antiviral, antifungal, antioxidant, anti-cancerous and immunomodulatory properties, but they can also be used as analgesic, anti-inflammatory, spasmolytic and local anesthetic remedies.[10]

The purpose of this study was to evaluate the efficacy of twelve EOs, extracted from Mediterranean plants, against forty-four strains, both methicillin-resistant and -susceptible, of *S. aureus*, isolated from different human clinical samples.

Correspondence: Nadia Massa, DiSIT - Università del Piemonte Orientale, viale T. Michel, 11, 15121 Alessandria;
E-mail: nadia.massa@uniupo.it
Tel.: +39.0131360231. Fax: +39.0131360243.

Key words: Staphylococcus aureus; essential oils; minimal inhibitory concentration; disk diffusion; fluorescein diacetate assay.

Conflict of Interest: The Authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

Further information: This work was partially presented to 42° Congress of the Italian Microbiology Society held in Turin with a poster presented by Giorgia Novello *et al,* titled '*In vitro* activity of twelve essential oils against clinical skin isolates *Staphylococcus aureus*'.

Received for publication: 2 October 2019.
Accepted for publication: 13 October 2019.


*Microbiology Research 2019; 10:8331*
*doi:10.4081/mr.2019.8331*

## Materials and Methods

### Microorganisms

*S. aureus* strains were collected by the Microbiology Departments of *SS. Antonio and Biagio and 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 rate 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 [Benzylpenicillin (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-sulphamethoxazole (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% and MH broth to a final concentration of 16% of the final volume. Serial doubling dilutions of oils (range 4% to 0.002% v/v) were prepared in a 96-wells microtiter plate. Strain suspensions obtained in physiological solution were adjusted to 0.5 McFarland by measuring absorbance at 600 nm. Growth control containing MH broth plus DMSO was also performed. All microtiter plates were incubated at 37 °C for 24 h. Each experiment was repeated three times.

### Fluorescein Diacetate Assay (FDA)

Fluorescein Diacetate Assay (FDA) (Sigma-Aldrich, St. Louis, MO, USA) stock solution (2.6 M) was prepared solving 1 g of fluorescein diacetate in 1 ml of sterilized potassium-phosphate buffer (8.7 g $K_2HPO_4$ and 1.3 g $KH_2PO_4$ 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): $\%FI = 100 - [(F_a/F_{max}) \times 100]$ where $F_a$ is the fluorescence of the assay (cells treated with EOs) and $F_{max}$ 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, 40.9% (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-

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

| Sample | Strain | Cefoxitin screening | MRSA* | BPC | OXA* | GEN | LVX | iR# to Clindamycin | iMLS§ | ERY | CLI | LZD | DAP | TEC | VAN | TET | TGC | FUS | RIF | SXT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference strain | NCTC 6571 | Negative | | 0.12 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 19 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Bronchial aspirate | 20 | Positive | MRSA | >=0 | >=4 | >=16 | 4 | Negative | | >=8 | >=4 | 2 | >=8 | <=0.5 | 1 | 2 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Bronchial aspirate | 21 | Negative | | >=0.5 | <=0.25 | <=0.5 | <=0.12 | Negative | | >=8 | >=4 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Bronchial aspirate | 22 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | >=4 | 2 | 1 | <=0.5 | >=32 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 23 | Negative | | 0.25 | <=0.25 | 8 | 0.25 | Negative | | >=8 | 0.25 | 2 | 0.25 | <=0.5 | 2 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 28 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | 0.25 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 31 | Negative | | >=0.5 | 0.5 | <=0.5 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Bronchial aspirate | 39 | Negative | | >=0.5 | <=0.25 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Throat swab | 40 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 41 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 1 | <=0.5 | <=0.5 | <=1 | 0.25 | <=0.5 | <=0.03 | <=10 |
| Blood culture | 53 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | 4 | Positive | iMLS | >=8 | <=0.12 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Throat swab | 54 | Negative | | >=0.5 | 0.5 | <=0.5 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 61 | Negative | | >=0.5 | 0.5 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | 0.25 | <=0.5 | >=4 | <=10 |
| Cutaneous swab | 62 | Negative | | >=0.5 | >=4 | <=0.5 | >=8 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Pus swab | 100 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 0.5 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 101 | Negative | | 0.06 | 0.5 | <=0.5 | 0.25 | Negative | | 0.5 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Nasal swab | 102 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 1 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Urine culture | 112 | Negative | | >=0.5 | 2 | <=0.5 | 0.5 | Negative | | 1 | 0.25 | 2 | 0.25 | 1 | 1 | <=1 | <=0.12 | 16 | <=0.03 | <=10 |
| Wound swab | 113 | Negative | | 0.06 | <=0.25 | >=16 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Vaginal swab | 114 | Positive | MRSA | >=0.5 | >=4 | 2 | >=8 | Negative | | >=8 | 0.25 | 2 | <=0.12 | 4 | 1 | 2 | 0.25 | <=0.5 | <=0.03 | >=320 |
| Throat swab | 115 | Negative | | >=0.5 | 0.5 | <=0.5 | <=0.12 | Negative | | 0.5 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Nasal swab | 116 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | 0.25 | <=0.5 | <=0.03 | <=10 |
| Pacemaker pocket swab | 141 | Negative | | >=0.5 | <=0.25 | <=0.5 | 0.25 | Negative | | 0.5 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 142 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 1 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 143 | Negative | | <=0.03 | <=0.25 | <=0.5 | <=0.12 | Negative | | 0.5 | 0.25 | 1 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Heel swab | 144 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Nasal swab | 145 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 1 | <=0.12 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Eye swab | 146 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | 4 | Positive | iMLS | >=8 | 0.25 | 2 | <=0.12 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 147 | Negative | | >=0.5 | 0.5 | <=0.5 | <=0.12 | Negative | | >=8 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 182 | Negative | | >=0.5 | 0.5 | <=0.5 | 1 | Negative | | 0.5 | 0.25 | 2 | 4 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 183 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | 1 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Ulcer swab | 184 | Negative | | >=0.5 | 0.5 | <=0.5 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Blood culture | 185 | Negative | | >=0.5 | <=0.25 | <=0.5 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 187 | Negative | | >=0.5 | <=0.25 | <=0.5 | <=0.12 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Tracheal aspirate | 188 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | 0.5 | Positive | iMLS | >=8 | 0.25 | 2 | 0.25 | <=0.5 | <=0.5 | >=16 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 189 | Negative | | >=0.5 | <=0.25 | 4 | 0.5 | Positive | iMLS | >=8 | 0.25 | 2 | <=0.12 | <=0.5 | 1 | >=16 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Wound swab | 190 | Positive | MRSA | >=0.5 | >=4 | <=0,5 | >=8 | Negative | | 0.5 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Nasal swab | 191 | Negative | | >=0.5 | <=0.25 | <=0.5 | <=0.12 | Negative | | 1 | <=0.12 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 223 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 224 | Positive | MRSA | >=0.5 | >=4 | >=16 | >=8 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 231 | Negative | | >=0.5 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.25 | 2 | 0.25 | <=0.5 | 1 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 232 | Negative | | 0.06 | 0.5 | <=0.5 | 0.25 | Negative | | 1 | 0.5 | 2 | 0.25 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 233 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Negative | | 1 | 0.25 | 2 | 0.5 | <=0.5 | <=0.5 | <=1 | 0.25 | <=0.5 | <=0.03 | <=10 |
| Cutaneous swab | 234 | Positive | MRSA | >=0.5 | >=4 | <=0.5 | >=8 | Positive | iMLS | >=8 | 0.25 | 2 | <=0.12 | <=0.5 | <=0.5 | <=1 | <=0.12 | <=0.5 | <=0.03 | <=10 |

BPC: Benzylpenicillin; OXA: 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; SXT:Trimethoprim-sulphamethoxazole; * 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.

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

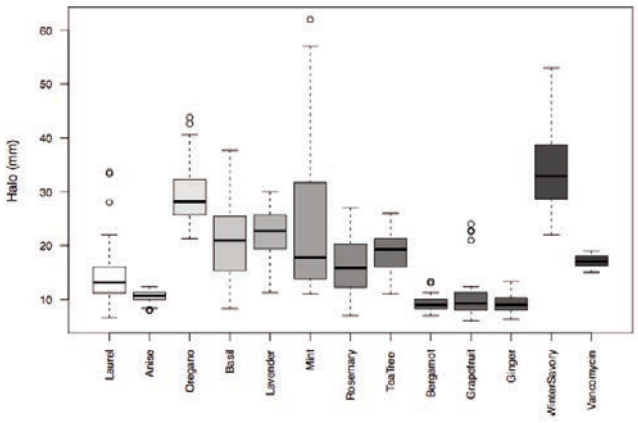


**Figure 1. Disk diffusion assay was carried out with 44 *S. aureus* strains and the reference strain *S. aureus* NCTC 6571. Vancomycin antibacterial effects were evaluated according to EUCAST Disk Diffusion Method for Antimicrobial Susceptibility v. 7.0 (January 2019). The sensitivity to the EOs was assessed using agar disk diffusion method.**

**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 | 4 | 1 | 4 | 1 | 4 | 2 | 1 | 0.0075 | 0.125 |
| 19 | | 0.5 | >4 | 2 | | | 1 | | 0.062 |
| 20 | | 1 | >4 | | | | 1 | | 0.125 |
| 21 | >4 | 0.5 | 4 | 2 | | | 2 | | 0.125 |
| 22 | | 0.5 | | | | | 1 | | 0.125 |
| 23 | | 1 | | | | | | | 0.125 |
| 28 | | 1 | 4 | | 4 | | | | 0.125 |
| 31 | | 1 | 4 | 2 | 4 | | | | 0.125 |
| 39 | | 0.5 | 4 | 2 | 4 | | 2 | | 0.25 |
| 40 | | 0.5 | | 2 | 4 | | | | 0.125 |
| 41 | | 1 | | 2 | 4 | | | | 0.125 |
| 53 | | 1 | 2 | 1 | 4 | 2 | 1 | | 0.125 |
| 54 | | 1 | 2 | 1 | 4 | | 1 | | 0.125 |
| 61 | | 0.5 | 1 | | 4 | | 1 | | 0.125 |
| 62 | >4 | 1 | | 1 | 4 | | 1 | | 0.125 |
| 100 | | 1 | 4 | | 4 | 2 | 1 | | 0.125 |
| 101 | >4 | 0.5 | 4 | 1 | | | | | 0.25 |
| 102 | >4 | 0.25 | >4 | 2 | | | 1 | | 0.125 |
| 112 | >4 | 0.25 | | | | | | | 0.062 |
| 113 | | 0.25 | >4 | | 4 | | | | 0.125 |
| 114 | | 0.25 | | | | | | | 0.125 |
| 115 | | 0.25 | >4 | 2 | | | | | 0.125 |
| 116 | | 0.25 | 4 | 2 | | 2 | | | 0.125 |
| 141 | >4 | 0.25 | >4 | 1 | | | 1 | | 0.25 |
| 142 | | 1 | >4 | 1 | | 2 | 2 | | 0.25 |
| 143 | | 0.25 | >4 | 2 | | | 1 | | 0.25 |
| 144 | | 0.25 | >4 | 2 | 2 | 2 | | | 0.25 |
| 145 | >4 | 0.25 | >4 | 2 | | | | | 0.25 |
| 146 | | 0.25 | | 2 | | 2 | 1 | | 0.25 |
| 147 | | 0.25 | >4 | 2 | | | 1 | | 0.25 |
| 182 | | 1 | | 2 | 4 | 2 | | | 0.25 |
| 183 | | 1 | | 2 | 4 | | 2 | | 0.25 |
| 184 | | 0.25 | | 2 | | | 2 | | 0.25 |
| 185 | >4 | 0.5 | >4 | 2 | 4 | | 1 | | 0.25 |
| 187 | | 0.25 | 4 | 2 | 2 | 2 | 1 | | 0.125 |
| 188 | | 1 | | 2 | 4 | | 1 | | 0.25 |
| 189 | | 1 | 2 | 2 | | 4 | 2 | | 0.25 |
| 190 | | 1 | | 2 | 4 | 2 | 1 | | 0.25 |
| 191 | | 1 | | 2 | | | | | 0.25 |
| 223 | | 1 | 4 | 2 | 2 | 2 | 1 | | 0.25 |
| 224 | | 0.5 | 4 | 0.25 | 2 | 2 | 1 | | 0.25 |
| 231 | | 1 | | 1 | 4 | | 1 | 0.125 | 0.25 |
| 232 | | 1 | 4 | 1 | 4 | | | 0.125 | 0.25 |
| 233 | | 0.5 | | 0.5 | | | | 0.125 | 0.25 |
| 234 | | 0.5 | | 1 | | | | 0.125 | 0.25 |

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.[12] Considering the current and still increasing problem of drug resistance,[13] 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.[14] 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

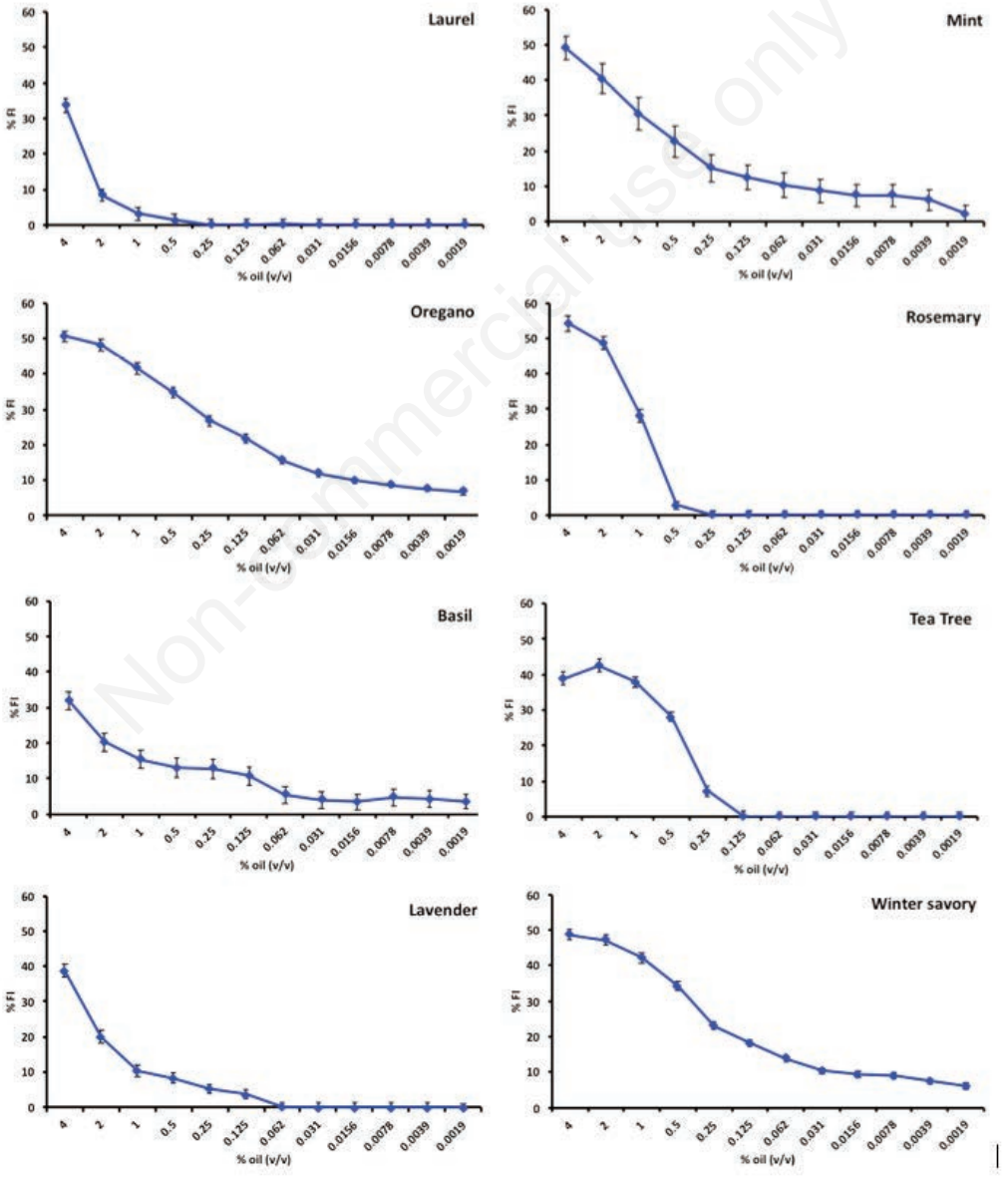


**Figure 2. The results for the reduction of the metabolic activity induced by decreasing concentrations of the different EOs, expressed as the mean values of data obtained for all the tested strains.**

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 coworkers[17] 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 physic-chemical 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 and Juliani[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 attribu-ted 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.

## References


1. de Kraker ME, Wolkewitz M, Davey PG, et al. Clinical impact of antimicrobial resistance in european hospitals: excess mortality and length of hospital stay related to methicillin-resistant Staphylococcus aureus bloodstream infections. Antimicrob Agents Chemother 2011;55:1598-605. doi:10.1128/AAC.01157-10.
2. Hoyert D, Xu J. Deaths: preliminary data for 2011. Natl Vital Stat Rep 2012;61:1-51.

3. Dall'Antonia M, Coen PG, Wilks M, et al. Competition between methicillin - sensitive and -resistant Staphylococcus aureus in the anterior nares. J Hosp Infect 2005;61:62-7. doi:10.1016/j.jhin.2005.01.008.
4. Waldvogel FA. Staphylococcus aureus (including toxic shock syndrome). In: Mandell GL, Bennett JE, Dolin R (eds) Mandell, Douglas, and Bennet's Principles and Practice of Infectious Diseases. 5th edn New York 2000:2069-92.
5. Koneman EW, Allen SD, Janda WM, et al. Testo-atlante di microbiologia diagnostica. 6th edn A.D. Editore 2009.
6. von Eiff C, Becker K, Machka K, et al. Nasal carriage as a source of Staphylococcus aureus bacteremia.Study Group. N Engl J Med 2001;344:11-6.
7. Bannerman TL, Peacock S. Staphylococcus, Micrococcus and other catalase-positive cocci. In: Murray PR, Baron EJ, Jorgensen JH, et al (eds). Manual of clinical microbiology, 9th edn. ASM Press 2007:390-411.
8. Muto CA, Jernigan JA, Ostrowsky, BE, et al. SHEA guideline for preventing nosocomial transmission of multidrug-resistant strains of Staphylococcus aureus infection. Infect Control Hosp Epidemiol 2003;24:362-86.
9. Baser, K.H.C., and Demitri, F. Chemistry of essential oils. In: Flavour and Fragrances: chemistry, bioprocessing and sustainability. Berger RG. (Ed). Springer 2007:43-86.
10. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils – A review. Food Chem Toxicol 2008;46:446-75. doi:10.1016/j.fct.2007.09.106.
11. Massa N, Cantamessa S, Novello G, et al. Antifungal activity of essential oils against azole-resistant and azole-susceptible vaginal Candida glabrata strains. Can J Microbiol 2018;64:647-63.
12. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. Ind Crop Prod 2014;62:250-64. doi:10.1016/ j.indcrop.2014.05.055.
13. Núnez-Núnez M, Navarro MD, Palomo V, et al. The methodology of surveillance for antimicrobial resistance and healthcare-associated infections in Europe (SUSPIRE ): a systematic review of publicly available information. Clin Microbiol Infect 2018;24:105-9. doi:10.1016/j.cmi.2017.07.014.
14. Gill AO, Delaquis P, Russo P, Holley RA. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. Int J Food Microbiol 2002;73:83-92. doi:10.1016/S0168-1605(01)00712-7.
15. May J, Chan CH, King A, et al. Time-kill studies of tea tree oils on clinical isolates. J Antimicrob Chemother 2000;45:639-43. doi:10.1093/jac/ 45.5.639.
16. Hada T, Furuse S, Matsumoto Y, et al. Comparison of the effects in vitro of tea tree oil and plaunotol on methicillin-susceptible and methicillin-resistant strains of Staphylococcus aureus. Microbios 2001;106:133-41.
17. Chao S, Young G, Oberg, C, Nakaoka. Inhibition of methicillin□resistant Staphylococcus aureus (MRSA) by essential oils. Flavour Fragr J 2008;23: 444-9 doi:10.1002/ffj.1904.
18. Marin M, Novacović M, Tešević V, et al. Antioxidative, antibacterial and antifungal activity of the essential oil of wild-growing Satureja montana L. from Dalmatia, Croatia. Flavour Fragr J 2012;27:216-23. doi:10.1002/ffj.3082.
19. Chedia A, Ghazghazi H, Dallali S, et al. Comparison of chemical composition, antioxidant and antimicrobial activities of Thymus capitatus L. essential oils from two tunisian localities (Sousse and Bizerte). Int J Agron Plant Prod 2013;4: 1772-81.
20. Hosni K, Hassen I, Chaâbane H, et al. Enzyme-assisted extraction of essential oils from thyme (Thymus capitatus L.) and rosemary (Rosmarinus officinalis L.): impact on yield, chemical composition and antimicrobial activity. Ind Crops Prod 2013;47:291-9. doi:10.1016/j.indcrop.2013.03.023.
21. Fabio A, Cermelli C, Fabio G, et al. Screening of the antibacterial effects of a variety of essential oils on microorganisms responsible for respiratory infections. Phyther Res 2007;21:374-7. doi:10.1002/ptr.1968.
22. Agarwal V, Lal P, Pruthi V. Prevention of Candida albicans biofilm by plant oils. Mycopathol 2008;165:13-9. doi:10.1007/s11046-007-9077-9.
23. Lang G, Buchbauer G. A review on recent research results (2008-2010) on essential oils as antimicrobials and antifungals. Flavour Fragr J 2012;27:13-39. doi:10.1002/ffj.2082.
24. Adukwu EC, Allen SCH, Phillips CA. The anti-biofilm activity of lemongrass (Cymbopogon flexuosus) and grapefruit (Citrus paradisi) essential oils against five strains of Staphylococcus aureus. J Appl Microbiol 2012;113: 1217-27. doi:10.1111/j.1365-2672.2012.05418.x.
25. Inouye S, Hideyo Y, Takizawa T. Screening of the antibacterial effects of a variety of essential oils on respiratory tract pathogens, using a modified dilution assay method. J Infect Chemother 2001;7:251-4.doi:10.1007/ s10156017 0022.
26. Carson CF, Mee BJ, Riley TV. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob Ag Chemother 2002;46: 1914-20. doi:10. 1128/AAC.46.6.1914–1920.2002.
27. Carson CF, Hammer KA, Riley TV. Melaleuca alternifolia (tea tree) oil: a review of antimicrobial and other medicinal properties. Clin Microbiol Rev 2006;19:50-62. doi:10.1128/CMR.19.1.50-62.2006.
28. Turina AV, Nolan MV, Zygadlo JA, Perillo MA. Natural terpenes: self-assembly and membrane partitioning. Biophys Chem 2006,122:101-13. doi:10.1016/j.bpc.2006.02.007.
29. Palmeira-de-Oliveira A, Salgueiro L, Palmeira-de-Oliveiro R, et al. Anti-Candida activity of essential oils. Mini Rev Med Chem 2009;9:1292-305.
30. Denyer SP, Hugo WB. Biocide-induced damage to the bac- terial cytoplasmic membrane. In: S. P. Denyer and W. B. Hugo (eds). Mechanisms of action of chemical biocides: their study and exploitation. Blackwell Scientific Publications Oxford 1991:171-87.
31. Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol 2001;91:453-62. doi:10.1046/j.1365-2672.2001.01428.x.
32. Ultee A, Bennik MHJ, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl Environ Microbiol 2002;68:1561-8.doi:10.1128/AEM.68.4.1561-1568.2002.
33 Burt S. Essential oils: their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol 2004;94:223-53. doi:10.1016/j.ijfoodmicro.2004.03.022.
34. Ipek E, Zeytinoglu H, Okay S, et al. Genotoxicity and antigenotoxicity of origanum oil and carvacrol evaluated by Ames Salmonella/microsomal test. Food Chem 2005;93:551-6. doi:10.1016/j.foodchem.2004.12.034.
35. Santiesteban-López A, Palou E, López-

Malo A. Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected $a_w$ and pH. J Appl Microbiol 2007;102:486-97. doi:10.1111/j.1365-2672.2006.03092.x.
36. Bassolé IHN, Juliani HR. Essential oils in combination and their antimicrobial properties. Molecules 2012;17:3989-4006. doi:10.3390/molecules17043989.
37. Juliani H, Koroch A, Simon J. Chemical diversity of essential oils of Ocimum species and their associated antioxidant and antimicrobial activity. In: Chemat F, Varshney VK, Allaf K (eds). Essential Oils and Aromas: Green Extractions and Applications. Har Krishan Bhalla & Sons: Dehradun, India, 2009.